

BOX PCT

ATTORNEY'S DOCKET NO: 24498

U.S. DEPARTMENT OF COMMERCE, PATENT AND TRADEMARK OFFICE		DATE: January 2001 (/18.01.2001)
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLN. NO. (if known): Not assigned <b>09/744016</b>
INTERNATIONAL APPLICATION NO.: PCT/EP99/05220	INTERNATIONAL FILING DATE: 22 July 1999 (22.07.99)	PRIORITY DATE CLAIMED: 22 July 1998 (22.07.98)
TITLE OF INVENTION: <b>METHOD FOR SCREENING OF MODULATORS OF CALCINERUIN ACTIVITY</b>		
APPLICANT(S) FOR DO/EO/US: VOELKEL, Helge		
Applicant hereby submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)):</p> <p style="margin-left: 40px;">a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</p> <p style="margin-left: 40px;">b. <input type="checkbox"/> has been transmitted by the International Bureau.</p> <p style="margin-left: 40px;">c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 40px;">a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</p> <p style="margin-left: 40px;">b. <input type="checkbox"/> have been transmitted by the International Bureau.</p> <p style="margin-left: 40px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 40px;">d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input checked="" type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>		
<b>ITEMS 11. TO 16. BELOW CONCERN OTHER DOCUMENT(S) OR INFORMATION INCLUDED:</b>		
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.		
12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.		
13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.		
<input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment		
14. <input type="checkbox"/> A substitute specification.		
15. <input type="checkbox"/> A change of power of attorney and/or address letter.		
16. <input checked="" type="checkbox"/> TRANSMITTAL FORM; FEE CALCULATION; INTERNATIONAL PUBLICATION WO 00/05363; INTERNATIONAL PUBLICATION DATE 03 FEBRUARY 2000 WITH ENGLISH TRANSLATION CONSISTING OF 130 PAGES INCLUDING: 1 COVER PAGE CONTAINING THE ABSTRACT; 42 PAGES TEXTUAL SPECIFICATION, 4 PAGES OF 23 CLAIMS; 0 SHEETS DRAWINGS; 83 PAGES SEQUENCE LISTING; PCT/ISA/210 INTERNATIONAL SEARCH REPORT; PCT/IPEA/409 INTERNATIONAL PRELIMINARY EXAMINATION REPORT WITH AMENDED SHEETS (CLAIMS) TO BE EXAMINED; PRELIMINARY AMENDMENT TO IPER AMENDED SHEETS; UNEXECUTED INVENTOR'S DECLARATION; ASSIGNMENT AND RECORDATION COVER SHEET; PCT/RO/101 REQUEST; PCT/IB/304 NOTIFICATION CONCERNING SUBMISSION OF PRIORITY DOCUMENT; PCT/IB/332 INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION; PCT IPEA/402 NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY.		

JC07 Rec'd PCT/PTO 19 JAN 2001

ATTORNEY'S DOCKET NO: 24498

U.S. APPLICATION NO. (if known) not yet assigned <b>09/744016</b>	INTERNATIONAL APPLICATION NO.  PCT/EP99/05220	DATE: January 2001 ( 18 .01.2001)
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17. x The following fees are submitted:

**Basic National Fee (37 CFR 1.492(a)(1)-(5):**

Search Report has been prepared by the EPO or JPO:.....\$860.00

International preliminary examination fee paid  
to USPTO (37 CFR 1.482).....\$690.00

No international preliminary examination fee paid  
to USPTO (37 CFR 1.482) but international search fee  
paid to USPTO (37 CFR 1.445(a)(2)).....\$710.00

Neither international preliminary examination fee  
(37 CFR 1.482) nor international search fee  
(37 CFR 1.445(a)(2)) paid to USPTO.....\$1000.00

International preliminary examination fee  
(37 CFR 1.482) and all claims satisfied provisions  
of PCT Article 33(2)-(4).....\$ 100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

CALCULATIONS

PTO USE ONLY

\$ 860.00

\$ 860.00

Surcharge of **\$130.00** for furnishing the oath or declaration later than    20  
   30 months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

CLAIMS	NO. FILED	NO. EXTRA	RATE		
TOTAL	<u>23</u> -20=	3	X \$ 18.00	\$	<b>54.00</b>
INDEPENDENT	<u>2</u> - 3=	1	X \$ 80 00	\$	0.00
Multiple dependent claims(s) (if applicable)			+ \$260.00	\$	0.00
TOTAL OF ABOVE CALCULATIONS =				\$	<b>914.00</b>
Reduction by 1/2 for asserting small entity, if applicable. (Note 37 CFR 1.9, 1.27, 1.28).				\$	<b>457.00</b>
SUBTOTAL =				\$	457.00
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <u>  </u> 20 <u>  </u> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	0.00
TOTAL NATIONAL FEE =				\$	457.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$	<b>40.00</b>
TOTAL FEES ENCLOSED =				\$	<b>497.00</b>
				Amount to be: refunded	\$
				charged	\$

ATTORNEY'S DOCKET NO: 24498

U.S. APPLICATION NO. (if known) not yet assigned  <b>09/744016</b>	INTERNATIONAL APPLICATION NO.  PCT/EP99/05220	DATE: January 2001 (18.01.2001)
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a. ☒ Two checks in the amount of \$497.00 to cover the above fees are enclosed.

b. ☐ Please charge my Deposit Account No. 14-0112 in the amount of \$\_\_\_\_\_ to cover the above fees. (A duplicate copy of this sheet is enclosed.)

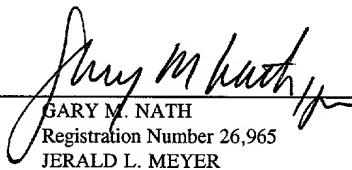
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0112.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed to request that the application be restored to pending status.

Send All Correspondence To:

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 GARY M. NATH  
 Registration Number 26,965  
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 Registration Number 41,194  
 Customer No. 20529

Rev. 02/98

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

JCU7 Rec'd PCT/PTO 19 JAN 2001

VOELKEL, Helge

International Application No. PCT/EP99/05220

Serial No. NOT YET ASSIGNED

International Filing Date: 22 July 1999 (22.07.99)

Filed: January 19, 2001

For: METHOD FOR SCREENING OF MODULATORS OF CALCINEURIN ACTIVITY

TRANSMITTAL LETTER

Commissioner of Patents

Washington, D.C. 20231

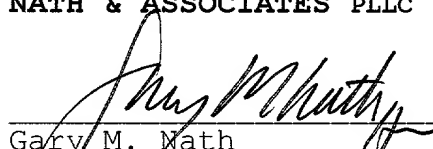
Sir:

Submitted herewith for filing in the U.S. Patent and Trademark Office is the following:

- (1) Transmittal Letter
- (2) Transmittal Letter To U.S. Designated/Elected Office (DO/EO/US) Concerning Filing under 35 U.S.C. 371
- (3) International Publication No: WO 00/05363  
International Publication Date: 3 February 2000 (03.02.00)  
which consists of 130 pages including:
  - 1 cover sheet containing the Abstract
  - 42 pages Textual Specification
  - 4 Pages of 23 claims
  - 0 Sheets of Drawings
  - 83 pages Sequence Listing
- (4) PCT/ISA/210 International Search Report
- (5) PCT/IPEA/409 International Preliminary Examination Report with Amended Sheets (claims) to be examined
- (6) Preliminary Amendment to IPER Amended Sheets
- (7) Unexecuted Inventor's Declaration
- (8) Assignment and Recordation Cover Sheet
- (9) PCT/RO/101 PCT Request
- (10) PCT/IB/304 Notification Concerning Submission of Priority Document
- (11) PCT/IB/332 Information Concerning Elected Offices Notified Cf Their Election
- (12) PCT/IPEA/402 Notification of Receipt of Demand by competent International Preliminary Examining Authority
- (13) Check No. 14086 \$457 .00 for Government Filing Fee as a small entity
- (14) Check No. 14087 \$ 40.00 for recordation fee
- (15) Postcard for early notification of serial number.

Respectfully submitted,  
**NATH & ASSOCIATES PLLC**

By:

  
Gary M. Nath  
Registration No. 26,965  
Jerald L. Meyer  
Registration No. 41,194  
Customer No. 20529

Date: January 19, 2001  
NATH & ASSOCIATES PLLC  
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GMN/JLM/dd:PCTappl.trans



BOX PCT-MISSING REQUIREMENTS  
Attorney Docket No. 24498

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:

VOELKEL, Helge

International Application No. PCT/EP99/05220

Serial No. 09/744,016

International Filing Date: 22 July 1999 (22.07.99)

Filed: January 19, 2001

For: **METHOD FOR SCREENING OF MODULATORS OF CALCINEURIN ACTIVITY**

**SUBMISSION OF MISSING REQUIREMENTS**

Commissioner for Patents  
Washington, D.C. 20231

Sir:

In response to the Notification of Missing Requirements Under 35 U.S.C. 371 mailed 9 March 2001, submitted herewith are the following:


- (1) Executed Declaration and Power of Attorney
- (2) Computer readable Form of the Sequence Listing
- (3) Verified Statement Claiming Small Entity Status
- (4) Two (2) Forms PCT/IB/306 Notification of the Recording of a Change
- (5) Check No. 14681 \$ 65.00 surcharge for late filing of Declaration as a small entity.

The Computer Readable Copy of the sequence listing is being submitted herewith on a 3.5" computer disk. As required by 37 CFR § 1.821(f), the Applicants hereby submit that the sequence information recorded on the enclosed computer disk is identical to the paper copy of the listings in the specification and includes no new matter.

The Commissioner is hereby authorized to charge any deficiency or credit any excess to Deposit Account No. 14-0112.

Respectfully submitted,  
**NATH & ASSOCIATES PLLC**

By: \_\_\_\_\_

  
Gary M. Nath  
Registration No. 26,965  
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Date: May 7, 2001

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09/744016

JC07 Rec'd PCT/PTO 19 JAN 2001

BOX PCT

Attorney Docket No. 24498

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:

VOELKEL, Helge

International Application No. PCT/EP99/05220

Serial No. NOT YET ASSIGNED

International Filing Date: 22 July 1999 (22.07.99)

Filed: January 19, 2001

For: **METHOD FOR SCREENING OF MODULATORS OF CALCINEURIN ACTIVITY**

**PRELIMINARY AMENDMENT**

Commissioner of Patents  
Washington, D.C. 20231

Sir:

Before calculating the filing fee for the above identified application, please enter the following amendments:

**IN THE CLAIMS:**

Claim 3, line 1, delete "or 2".

Claim 4, line 1, delete "or 2".

Claim 5, line 1, delete "one of the preceding claims" and insert lieu thereof --claim 1--.

Claim 6, line 1, delete "one of the preceding claims" and insert lieu thereof --claim 1--.

Claim 8, line 1, delete "one of the preceding claims" and insert lieu thereof --claim 1--.

Claim 9, line 1, delete "one of the preceding claims" and insert lieu thereof --claim 1--.

Claim 10, line 1, delete "one of the preceding claims" and insert lieu thereof --claim 1--.

Claim 13, line 1, delete "one of the preceding claims" and insert lieu thereof --claim 1--.

Claim 14, line 1, delete "one of the preceding claims" and insert lieu thereof --claim 1--.

Claim 17, line 1, delete "or 16"

Claim 18, line 1, delete "one of claims 3 to 17" and insert in lieu thereof --claim 3--.

Claim 19, line 2-3, delete "one of the preceding claims" and insert lieu thereof --claim 1--.

Claim 21, line 1, delete "or claim 20"

Claim 22, line 1, delete "one of claims 19 to 21" and insert lieu thereof --claim 19--.

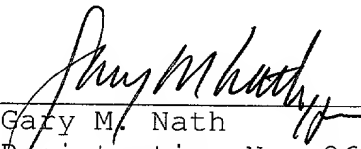
**REMARKS**

The above amendments have been made to remove multiple dependencies from the claims, and no new matter has been added.

Respectfully submitted,

**NATH & ASSOCIATES PLLC**

By: \_\_\_\_\_

  
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Date: January 19, 2001  
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Washington, D.C. 20005  
GMN/JLM/dd:AMENDprempCT



PCT09

## RAW SEQUENCE LISTING

DATE: 02/14/2002

PATENT APPLICATION: US/09/744,016A

TIME: 10:12:44

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3 <110> APPLICANT: Dr. Voelkel, Helge  
 5 <120> TITLE OF INVENTION: Method for screening of modulators of calcineurin  
 6 activity  
 8 <130> FILE REFERENCE: A34157PCT  
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 C--> 11 <141> CURRENT FILING DATE: 2001-10-03  
 13 <150> PRIOR APPLICATION NUMBER: EP98113876  
 14 <151> PRIOR FILING DATE: 1998-07-22  
 16 <160> NUMBER OF SEQ ID NOS: 35  
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 25 <220> FEATURE:  
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 32 <222> LOCATION: (1124)..(1849)  
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 62 ggc atc atc aat ttc gag cag aag gaa agt aat gga cca gtg aag gtg 757  
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 66 tgg gga agc att aaa gga ctg act gaa ggc ctg cat gga ttc cat gtt 805  
 67 Trp Gly Ser Ile Lys Gly Leu Thr Glu Gly Leu His Gly Phe His Val  
 68 35 40 45

5 Method for screening of modulators of calcineurin activity**Specification:**

10 The invention relates to a method for screening of modulators of calcineurin.

Calcineurin (E.C. 3.1.3.16) is a serine/threonine phospho-  
protein phosphatase and is composed of a catalytic (calcineu-  
rin A) and regulatory (calcineurin B) subunit (about 60 and  
15 about 18 kDa, respectively). In mammals, three distinct genes  
(A-alpha, A-beta, A-gamma) for the catalytic subunit have  
been characterized, each of which can undergo alternative  
splicing to yield additional variants. Although mRNA for all  
three genes appears to be expressed in most tissues, two  
20 isoforms (A-alpha and A-beta) are most predominant in brain.

Calcineurin has been cloned from various organisms including  
human (Guerini et al., 1989), (Guerini and Klee, 1989),  
(Kincaid et al., 1991), (Kuno et al., 1989), (Ito et al.,  
25 1989), (Muramatsu and Kincaid, 1993). The crystal structure  
has shown that calcineurin A contains a binuclear metal  
center with unknown enzymatic function (Griffith et al.,  
1995). Recombinant expression of rat calcineurin A subunit in  
bacteria or SF9-cells were not effective and yielded only  
30 poor enzymatic activities since calcineurin A is not stable  
in the absence of calcineurin B (Perrino et al., 1992),  
(Perrino et al., 1995), (Haddy and Rusnack, 1994). Coexpres-  
sion of calcineurin A and calcium binding subunit calcineurin  
B yielded a more stable and active enzyme (Mondragon et al.,

1997). Calcineurin has been implicated in various neuronal signaling pathways (Klee et al., 1988), (Yakel, 1997) but the neuronal function is only poorly understood (Guerini, 1997).

5 Calcineurin is the only protein phosphatase known to be under the control of  $\text{Ca}^{2+}$  and calmodulin. Binding of  $\text{Ca}^{2+}$  and calmodulin is necessary for enzymatic activity. Calmodulin is bound by the catalytic subunit whereas the regulatory subunit possesses four  $\text{Ca}^{2+}$  binding sites.

10 Calcineurin is discussed in the context of immunosuppression. It has been shown that calcineurin acts via the transcription factor NFAT (nuclear factor of activated T cells) on the T cell response. The functions of NFAT proteins are directly  
15 controlled by calcineurin in a calcium- and calmodulin-dependent manner. Activation of NFAT by calcineurin is mediated by the cytosolic binding protein FKBP.

Substances which are able to block the calcineurin signal  
20 pathway are suitable agents in order to block the T cell activation and thereby suppressing the immune response. Suppression of immune response has important clinical relevance, for example in transplantation surgery for preventing rejection episodes. Therefore, calcineurin as pharmacological  
25 target is of great importance and several attempts were made to develop agents which block the calcineurin signal pathway. Examples of such immunosuppressive drugs are FK506 (Fujisawa) and cyclosporine (Novartis) (Liu et al, 1991). These  
30 antibiotics inhibit calcineurin phosphatase activity in the presence of immunophilin receptor proteins (FKBP, cyclophilin) and thereby suppress immune response by preventing the activation of the T cell transcription factor NFAT (Liu et al., 1992), (Nelson et al., 1993). FK506 (tacrolimus) binds to the binding protein FKBP and thereby prevents calcineurin

from binding to FKBP. Accordingly the signal pathway is interrupted. No activation of the transcription factor NFAT is achieved and the T cell activation is disturbed.

5 Nevertheless, there are several severe disadvantages and side-effect of said drugs. In clinical trials with liver and renal transplant recipients it has been shown that FK506-based therapy was associated with increased toxicities in comparison to conventional therapy. Furthermore FK506 has  
10 negative effects on the bone mineral physiology.

Besides the role of the calcineurin signal pathway in immune response it has been shown that calcineurin is involved in apoptosis induction by glutamate excitotoxicity in neuronal  
15 cells (Ankarcrona et al., 1996). Low enzymatic levels of calcineurin have been associated with Alzheimers disease (Ladner et al., 1996), (Kayyali et al., 1997). Calcineurin inhibitors (FK506, Cyclosporin) prevented epileptogenesis in model organisms (Moriwaki et al., 1996). In the heart or in  
20 the brain calcineurin also plays a key role in the stress response after hypoxia or ischemia (Butcher et al., 1997), (Hashimoto et al., 1998), (Molkentin et al., 1998).

In summary, calcineurin is a crucial target to develop new  
25 substances suitable as drugs, especially as immunosuppressive drugs. Former screening systems using purified calcineurin and conventional assays like radioactive or HPLC assays (Klee, 1991), (Enz et al., 1994) did not lead to appropriate new substances. Therefore, the invention has the object to  
30 provide a new screening system for modulators of calcineurin taking advantage of new insights into the signal pathway of calcineurin. By the use of this new screening system it is possible to develop new pharmaceuticals with respect to the field of transplantation surgery, cardiac infarction and

apoplexy, chronic or acute neurodegeneration and inflammatory diseases, for example. This object is solved by a method according to claim 1. Preferred embodiments of the inventive method are depicted in the dependent claims 2 to 17. A kit,  
5 vectors, cells and a peptide suitable for performing the inventive method are claimed in claim 18 to 23. The wording of all claims is hereby made to the content of the specification by reference.

- 10 The inventive method is based on results showing that a physiological interaction between calcineurin and superoxide dismutase takes place which provides a suitable target for developing of a new screening system.
- 15 For a long time it was not understood why recombinant or even purified calcineurin exhibited only 1 to 2 % of the specific activity estimated in crude brain extracts until it was detected that the binuclear metal center of the enzyme contains a redoxsensitive  $\text{Fe}^{2+}$  (Yu et al., 1997). After  
20 calcium activation or during purification procedure the  $\text{Fe}^{2+}$  is oxidized by oxygen species and turns the enzyme inactive (Stemmer et al., 1995), (Wang et al., 1996).

Recently it has been shown that copper/zinc superoxide  
25 dismutase (CuZnSOD, EC 1.15.1.1) protects calcineurin against oxidative inactivation (Wang et al., 1996). The phosphatase activity of calcineurin is strongly dependent on the presence of calcium and calmodulin. The addition of  $\text{Ca}^{2+}$  in the presence of calmodulin leads to a drastic increase in activi-  
30 ty. But during several minutes this activity is lost. By the addition of copper/zinc superoxide dismutase the activity can be maintained.



Superoxide dismutase (SOD) dismutates the hyperoxide anion (superoxide) into hydroperoxide and molecular oxygen. There are two forms of this enzyme: the mitochondrial form containing manganese and the cytosolic form containing copper and zinc. In general superoxide dismutase is considered to be a catcher of radicals and is discussed in the field of detoxification of reactive oxygen species. Therefore, the role of superoxide dismutase in the protection of the activity of calcineurin found by Wang et al. was considered to be the result of general redox function of superoxide dismutase. Now, surprising results of the inventor lead to the knowledge that a physiological interaction between calcineurin and superoxide dismutase takes place. Several mutants of copper/zinc superoxide dismutase lacking the enzymatic function showed the protective effect on the activity of calcineurin. That means that the effect of CuZnSOD is not due to the function of superoxide dismutase in redox regulation. These results teach that superoxide dismutase interacts physiologically with calcineurin and that CuZnSOD is one component of the calcineurin pathway which is important for the physiological functions of calcineurin.

These results are used to develop a new screening system for modulators of calcineurin in order to find inhibitors or activators of the calcineurin signal pathway. The inventive method is based on the complex formation between calcineurin and superoxide dismutase in the presence of potential modulators of this physiological interaction. If a potential modulator disturbs the complex formation, this substance is a good candidate for inhibiting the calcineurin signal pathway and could possibly be used as immunosuppressive drug, for example. On the other hand it could be favourable to identify a substance which promotes complex formation and thereby stimulates the calcineurin signal pathway, e.g. the T cell

response in result. Such a substance could be used in order to strengthen immune response. By the term "modulator" is meant any substance which influences the complex formation relating to the inventive method. Additionally is meant any substance which influences the interaction between calcineurin and its substrates, e.g. the peptide RII. Furthermore is meant any substance which influences the superoxide dismutase and/or calcineurin on the transcriptional, the translational and/or the posttranslational level.

Calcineurin as used in the inventive method is build up by the regulatory subunit A and the catalytic subunit B. The presence of both subunits is essential for physiological activity of calcineurin. Nevertheless, it is possible to perform the inventive method using only one of the subunits. There are several isoforms of calcineurin consisting of subunit calcineurin B and one out of the group comprising subunit calcineurin A-alpha, A-beta and A-gamma. Each isoform represents a special cell and tissue specific distribution. Therefore, the choice of isoform could be crucial for cell and tissue specificity of the substance to be screened. With respect to clinical application of the substances to be screened preferably human forms of the proteins are used.

Furthermore it is preferred to perform the inventive method in the presence of calmodulin and calcium, because the activity of calcineurin is dependent on these factors. Preferably the cytosolic form of superoxide dismutase containing copper and zinc is used for complex formation, because interaction between the mitochondrial form of superoxide dismutase containing e.g. manganese normally does not occur under physiological conditions. The complex formation is performed in the presence of at least one potential modulator of calcineurin or the calcineurin signal pathway, respec-

tively. The complex comprising calcineurin A, calcineurin B, superoxide dismutase and preferably calmodulin is the target for potential modulators which could stabilize or disturb the complex.

5

Advantageously, the complex formation is monitored during the whole process. It is possible to add the modulator before or after the complex formation has been performed. Preferably the modulator is added before complex formation because the effect of a weak modulator will possibly not be monitorable when complex formation has already finished.

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In principle, there are two possibilities to monitor the complex formation. Firstly, the complex formation is directly monitored by the use of labeled components in the complex, preferably by fluorescence detection. Secondly, the complex formation is monitored by the activity of the complex, especially the enzymatic activity of calcineurin. This second method can be performed in addition to the firstly mentioned method or as an alternative. Clearly, the inventive method is not restricted by the method for detecting the influence of the modulator on complex formation.

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In a preferred embodiment of the invention, a mixture of substances comprising at least one potential modulator is analyzed by the inventive method. By isolating the complex together with the possibly interacting modulator it is possible to separate the modulator out of the mixture and to identify it by common methods.

In one preferred embodiment of the invention the calcineurin and/or superoxide dismutase are labeled. Especially preferred is the use of fluorescent labels. Preferably, the labeled proteins are fusion proteins comprising a fluorescent prote-

in, e.g. enhanced green fluorescent protein (EGFP). These fusion proteins are provided by genetic engineering methods. It is also possible to label said proteins by other methods known to experts in the art, e.g. by the use of radioactive isotopes which are incorporated into the proteins.

Advantageously the components of the complex, i.e. calcineurin and superoxide dismutase are expressed in the cell, especially in an eukaryotic cell, as fluorescent fusion proteins. By the use of laser fluctuation correlation spectroscopy the complex formation of labeled proteins is monitored directly within the cell. This embodiment of the invention is described in greater detail in the example. The invention comprises several vectors useful for the expression of calcineurin and/or superoxide dismutase in eukaryotic cells. These vectors encode the proteins, especially CuZnSOD and the different subunits of calcineurin, as fusion proteins in connection with the fluorescent protein EGFP (enhanced green fluorescent protein). EGFP is only one example of possible labels useful in respect of the inventive method. Furthermore, the invention comprises cells, especially eukaryotic cells, stably transfected with the above-mentioned vectors thereby expressing superoxide dismutase and/or calcineurin. Preferably, these proteins are coexpressed, i.e. expressed within the same cell.

In an especially preferred embodiment, the genetic information of fusion proteins is integrated in the cell by homologous recombination. That means that the gene encoding the recombinant protein, especially the fluorescent fusion protein, is incorporated in the genome of the cell in the place of the naturally occurring gene. This leads to a cell essentially lacking the natural protein. By the use of such cells it is possible to identify modulators by the inventive method which

influence the transcriptional, translational or posttranslational level of calcineurin and/or superoxide dismutase expression.

5 In another embodiment of the inventive method the components of the complex are isolated and preferably purified before complex formation is performed in vitro. Advantageously, the proteins are provided with a tag in order to facilitate purification, e.g. a histidin (his) tag consisting of several  
10 histidines in sequence which permits affinity purification by known procedures. Corresponding vectors encoding the tagged proteins are comprised by the invention. These vectors are especially useful as prokaryotic expression vectors. Furthermore, the invention comprises cells bearing said vectors.

15 Advantageously, following purification of the his-tagged proteins the tag is excised by appropriate enzymatic digestion, e.g. by the use of cathepsin-C or carboxypeptidase-A. Especially preferred is the purification of calcineurin by  
20 ferro-nitrilotriacetat-metal (Fe-NTA) affinity chromatography and the purification of superoxide dimutase by copper/-zinc-nitrilotriacetat-metal (CuZn-NTA) affinity chromatography. Nevertheless, other purification procedures known to experts in the art are possible. Natural occurring protein  
25 could also be used in the inventive manner.

Besides the use in purification of calcineurin and/or superoxide dismutase Ni (nickel)-NTA, Fe-NTA and/or Cu/Zn-NTA is used to immobilize the his-tagged calcineurin and/or super-  
30 oxide dismutase in order to isolate naturally occurring ligands of these proteins using this inventive matrix. By the term "ligand" is meant any low- or highmolecular endogenous, exogenous or synthetic substance which interacts with said proteins. This could be a peptide, protein, carbohydrate,

lipid, nucleic acid or a synthetic polymer, for example. These so-identified ligands are potential candidates for modulators of the calcineurin signal pathway.

- 5 When performing complex formation in vitro it could be preferred to add calmodulin and/or calcium to the reaction because these factors are necessary for enzymatic activity of calcineurin.
- 10 In another preferred embodiment of the invention the complex formation is monitored indirectly by analyzing the enzymatic activity of calcineurin. As outlined above the phosphatase activity of calcineurin is strictly dependent on the interaction with superoxide dismutase. Therefore, it is possible to
- 15 monitor the complex formation indirectly by the measurement of phosphatase activity according to standard procedures. This is especially preferred if the laboratory equipment to perform fluorescent measurements as described above is not available. Furthermore, enzymatic analysis could be used in
- 20 addition to fluorescence detection like laser fluctuation correlation spectroscopy, e.g. as control.

Preferably the enzymatic activity is analyzed by the use of a labeled substrate of calcineurin. The substrate is preferably

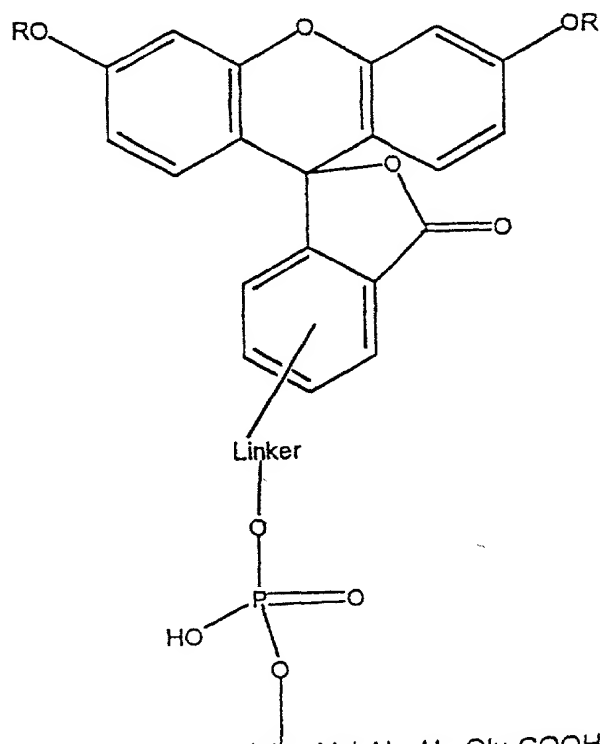
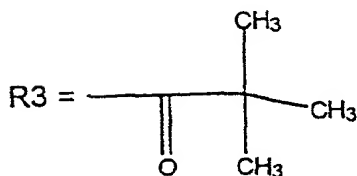
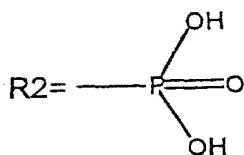
25 labeled by fluorescence. One especially preferred substrate is the peptide RII characterized by the sequence:

Asp - Leu - Asp - Val - Pro - Ile - Pro - Gly - Arg -  
Phe - Asp - Arg - Arg - Val - Ser - Val - Ala - Ala -  
30 Glu.

In a preferred embodiment this peptide carries a fluorescent label at serine in position 15. This amino acid is

labeled with fluoresceine by incubating the peptide with fluoresceine-phosphoamidit, thereby providing a labeled substrate (RII-Fluophos). RII interacts with the active center of calcineurin, but it is not converted by the phosphatase. Hereby it is possible to label calcineurin in the active state. Furthermore, it is possible to phosphorylate RII-Fluophos at the fluoresceine moiety as depicted below at tyrosine residues. Due to the phosphorylation RII-Fluophos loses its fluorescence and thereby provides a phosphatase substrate which becomes fluorescent subsequent to dephosphorylation.

R1 = H



-NH<sub>2</sub>-Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Ala-Ala-Glu-COOH-

This peptide could be provided synthetically or it is expressed by cells, especially eucaryotic cells, which have been transfected with appropriate vectors encoding said peptide or other peptides useful as phosphatase substrates. In one  
5 embodiment of the invention the fluorescent peptide is used as peptide label in fluorescence microscopy. This provides another method in order to analyze the active state of the calcineurin/superoxide complex.

10 The inventive method as outlined above is suitable for developing a high-throughput bioassay to identify inhibitors and/or activators of the calcineurin signal pathway. Details are described in the example.

15 The invention comprises the use of inhibitors of activators of the calcineurin signal pathway for the treatment of acute and/or chronic neurological and cardiovascular diseases like Alzheimer, Parkinson, epilepsy, ischemia and heart-failure. Furthermore the use as immunosuppressive drugs, e.g. in the  
20 field of transplantahtation surgery and inflammatory diseases is included.

Finally the invention comprises a kit for screening of modulators of calcineurin. The kit provides calcineurin and  
25 superoxide dismutase enabling complex formation for the screening for modulators of calcineurin as described above. In a first embodiment of the kit the components of the complex are provided as proteins. This kit is suitable for performing the inventive method in vitro. In a second embodi-  
30 ment of the kit the proteins are provided in the form of vectors. These vectors have to be transformed/transfected into cells leading to the expressed proteins. These vectors are prokaryotic or eukaryotic expression vectors, respectively, and could be used to produce the proteins for the in



vitro assay or for the assay using complete cells as described above. In a third embodiment of the inventive kit cells transformed/transfected with the said vectors are provided saving the step of transforming/transfecting for the user.

5 For details of the inventive kit reference is made to the above description.

The new approach to identify new substance classes of calcineurin/CuZnSOD inhibitors comprises inter alia:

10

- coexpression of CuZnSOD/calcineurin A and calcineurin B to generate a oxidative stable enzyme which is suitable for drug screening,

15

- efficient purification of CuZnSOD on CuZn-nitrilotriacetat-metal affinity chromatography to retain enzymatic activity,

20

- efficient purification of calcineurin on Fe-nitrilotriacetat-metal affinity chromatography to retain enzymatic activity and prohibit  $\text{Fe}^{2+}$  oxidation,
- identifying that mutations in the CuZnSOD associated with a neurological disorder (amyotrophic lateral sclerosis) are also critical for calcineurin-CuZnSOD interaction,

- use of fluorescent labeled recombinant CuZnSOD and calcineurin to screen for CuZnSOD/calcineurin activators or

25 inhibitors,

- use of fluorescent labeled RII-peptide and calcineurin to screen for calcineurin activators or inhibitors,

- identification of calcineurin/CuZnSOD inhibitors or activators by using the recombinant enzymes as affinity ligands to

30 purify new drugs from natural sources,

- inclusion of all isoforms, all known and two newly identified splicevariants into the screening procedure, which allows the identification of less toxic and tissue specific

drugs which are more suitable for the therapeutical treatment of different clinical indications.

The described features of the invention and further features result in greater detail from the examples in combination with the subclaims. The features could be realized in combination with each other or alone.

#### Example

##### 1. Cloning of CuZnSOD transcript from human brain poly-A-RNA -

Cloning of human CuZnSOD was performed by reverse transcription PCR using human brain poly-A-RNA as template (Clontech, Palo Alto, CA, USA). The oligonucleotides SODs1 5'-ttc cgt tgc agt cct cgg aac-3', SODas1 5'-taa ggg gcc tca gac tac atc-3', SOD-PQE60s2 5'-caa gcc atg gcg acg aag gcc gtg tgc gtg ctg-3', SOD-PQE60as2 5'-gaa gat ctt tgg gcg atc cca att aca cca c-3', SOD-PQE30-s2 5'- cgc gga tcc gcg acg aag gcc gtg tgc gtg -3' and SOD-PQE30-as2 5'-ggg ttc gaa tta ttg ggc gat ccc aat tac-3' were supplied by Interactiva (Ulm, Germany). Reverse transcription was performed with the SODas1 primer and 100 ng of poly-A-RNA according to the manufacturer's protocol (Expand reverse transcriptase, Boehringer Mannheim, Germany). The human CuZnSOD cDNA was amplified by nested PCR. The first PCR was performed in 20  $\mu$ l, using 0,5  $\mu$ l reverse transcription product, 10  $\mu$ M SODs1 and SODas1 primers, 300  $\mu$ M dNTPs, 2  $\mu$ l of the manufacturer's 10 x PCR buffer and 2.5 U Taq-polymerase with 30 cycles of 1 min 95  $^{\circ}$ C, 1 min 45  $^{\circ}$ C, 1 min 72 $^{\circ}$ C followed by a second PCR (50  $\mu$ l) with 5  $\mu$ l of the purified first PCR product, 10  $\mu$ M SOD-PQE60s2 and SOD-PQE60as2 primers, 300  $\mu$ M dNTPs, 5  $\mu$ l of the manufacturer's PCR buffer and 2,5 U Taq-polymerase with 30 cycles of 1 min 95  $^{\circ}$ C, 1 min 60  $^{\circ}$ C, 1 min 72 $^{\circ}$ C (Taq-polymerase, Pharmacia Biotech, Uppsala, Sweden). For the subcloning

into pQE30 expression vector the primers SOD-pQE30-s2 and SOD-pQE30-as2 were used instead of SOD-pQE60s2/SOD-pQE60as2.

## 2. Subcloning of human CuZnSOD into pQE60 expression vector

5 (C-terminal fusion protein) - The SOD-pQE60 PCR product was purified by gel extraction prior to NcoI/BglII restriction (New England Biolabs). In order to generate a C-terminal histidin tag fusion protein the CuZnSOD transcript was ligated into the NcoI/BglII treated prokaryotic expression  
10 vector pQE60 (QIAexpress expression kit type IV and type ATG, Qiagen, Hilden, Germany). For selection, amplification and sequencing of the CuZnSOD vector construct (CuZnSOD-pQE60), 10 µl ligation product was transformed into E.coli M15[pREP4] cells (QIAexpress expression kit type ATG, Qiagen, Hilden,  
15 Germany). Correct reading frames and exclusion of mismatches were confirmed by radioactive and automated sequencing on both strands (T7-sequencing kit, Pharmacia Biotech, Uppsala, Sweden; ABI 377 sequencer, Applied Biosystems, USA).

## 20 3. Subcloning of human CuZnSOD into pQE30 expression vector

(N-terminal fusion protein) - The SOD-pQE30 PCR product was purified by gel extraction prior to direct ligation into the pCR2.1 vector according to the manufacturer's protocols (TA-Cloning Kit, Invitrogen, De Schelp, Netherlands). After  
25 amplification and plasmid purification the pCR2.1-CuZnSOD vector construct was restricted with BamHI to yield a CuZnSOD transcript extended at the 3'-end with the sequence 5'-GAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCC-3' which originates from PCR2.1 vector and includes additional EcoRI/BstX-I/  
30 SpeI/BamHI restriction sites. In order to generate a N-terminal histidin tag fusion protein the extended transcript was ligated into the BamHI/HindIII treated prokaryotic expression vector pQE30 (QIAexpress expression kit type IV, Qiagen, Hilden, Germany), blunted by incubation with Klenow-DNA-poly-

merase and circularized by a second treatment with T4-DNA-Li-gase (Boehringer Mannheim, Germany). For selection, amplification and sequencing of the CuZnSOD vector construct (CuZn-SOD-pQE30), 10  $\mu$ l ligation product was transformed into

- 5 E.coli M15[pREP4] cells (QIAexpress expression kit type IV and type ATG, Qiagen, Hilden, Germany). Correct reading frames and exclusion of mismatches were confirmed by radioactive and automated sequencing on both strands (T7-sequencing kit, Pharmacia Biotech, Uppsala, Sweden; ABI 377 sequencer, 10 Applied Biosystems, USA).

**4. Site directed mutagenesis (point mutations associated with the neurological disorder Amyotrophic Lateral Sclerosis and important for calcineurin/CuZnSOD protein interaction) -**

- 15 Amino acid substitutions were introduced according to the manufacturer's protocol, using the primers SOD-PQE60-A4V (5'-caa gcc atg gcg acg aag gtc gtg-3'), SOD-A4V (5'-tcc gcg acg aag gtc gtg tgc gtg ctg-3'), SOD-G37R (5'-gg aag catt aaa aga ctg act gaa ggc-3'), SOD-D90A (5'-aat gtg act gct gcc aaa gat ggt gtg-3'), SOD-G93A (5'-gct gac aaa gat gct gtg gcc gat gtg-3'), SOD-AflIII (5'-acg cag gaa aga aca tgt gag caa aag-3'), SOD-BglIII (5'-acg cag gaa aga aga tct gag caa aag-3') and the expression vector CuZnSOD constructs CuZn-SOD-pQE30 and CuZnSOD-pQE60, respectively (Chameleon site 20 directed mutagenesis kit, Stratagene, San Diego, CA, USA). Incorporation of the site-directed mutations was confirmed by DNA sequencing of the expression vector. Site directed mutagenesis yielded eight additional vector sequences corresponding to eight protein sequences with clinical relevant 30 amino acid substitutions:

Vector-construct	amino acid subst. (pos. in protein)	nucleic acid subst. (pos. in sequ. prot.)
CuZnSOD-pQE60	WT = wild-type	= SEQ ID NO 15

CuZnSOD-pQE60-(A4V)	Ala-4	-> Val-4	c-128	-> t-128
CuZnSOD-pQE60-(G37R)	Gly-37	-> Arg-37	g-226	-> a-226
CuZnSOD-pQE60-(D90A)	Asp-90	-> Ala-90	a-386	-> c-386
CuZnSOD-pQE60-(G93A)	Gly-93	-> Ala-93	g-395	-> c-395

5

CuZnSOD-pQE30	WT = wild-type = SEQ ID NO 13			
CuZnSOD-pQE30-(A4V)	Ala-4	-> Val-4	c-161	-> t-161
CuZnSOD-pQE30-(G37R)	Gly-37	-> Arg-37	g-259	-> a-259
CuZnSOD-pQE30-(D90A)	Asp-90	-> Ala-90	a-419	-> c-419
10 CuZnSOD-pQE30-(G93A)	Gly-93	-> Ala-93	g-428	-> c-428

#### 5. Recombinant expression and purification of wild-type and mutated CuZnSOD

The CuZnSOD-pQE60 or CuZnSOD-pQE30 vector transformed E.coli M15[pREP4] cells were plated on LB /

15 ampicillin (100 µg/ml) / kanamycin (25 µg/ml) agar. Expression cultures were grown in 250 ml LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) until the OD<sub>600</sub> was 0.6.

Constitutive leakage expression of human CuZnSOD was fully prevented by the repressor plasmid pREP4-lacI. Production of

20 the human CuZnSOD fusion proteins was induced by addition of IPTG (1 mM). After two hours the bacterial cells were harvested by centrifugation (4000 g, 20 min), resuspended in 8 ml buffer A (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM

NaCl) and homogenized by three freeze thaw cycles and sonication on ice (Bandelin sonoplus GM70, 300 W, 3 x 10 sec). The lysate was centrifuged (10.000 g, 20 min) and incubated with 750 µl CuZn-NTA (nitrilotriacetat)-agarose for batch affinity binding for 1 h at 4 °C (Qiagen expressionist kit, Qiagen, Hilden, Germany). CuZn-NTA-agarose was prepared from Ni-

30 NTA-agarose (Qiagen expressionist kit, Qiagen, Hilden, Germany) by subsequent washes in:

1) 2 volumes of bidistilled water

2) 3 volumes of regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)

- 3) 5 volumes bidestilled water  
4) 3 volumes 2% SDS  
5) 1 volume 25% ethanol  
6) 1 volume 50% ethanol  
5 7) 1 volume 75% ethanol  
8) 5 volumes 100% ethanol  
9) 1 volume 75% ethanol  
10) 1 volume 50% ethanol  
11) 1 volume 25% ethanol  
10 12) 1 volume bidestilled water  
13) 5 volumes 100 mM Na-EDTA pH 8.0  
14) 5 volumes bidestilled water  
15) 2 volumes 100 mM CuSO<sub>4</sub> / 100 mM ZnSO<sub>4</sub> / 1 mM reduced  
glutathion / 1 mM dithiothreitol  
15 16) 2 volumes bidestilled water  
17) 2 volumes regeneration buffer (6 M guanidiniumhydrochlo-  
ride, 0.2 M acetic acid)  
18) 2 volumes buffer buffer A2 (20 mM Tris-HCl pH 7.9, 5 mM  
imidazole, 500 mM NaCl, 200 µM CuSO<sub>4</sub> / 200 µM ZnSO<sub>4</sub> / 1 mM  
20 reduced glutathion / 1 mM dithiothreitol

The batch was applied to a 30 ml chromatography column,  
washed with 15 ml buffer A (20 mM Tris-HCl pH 7.9, 5 mM  
imidazole, 500 mM NaCl) and subsequently with 8 ml buffer B  
25 (20 mM Tris-HCl pH 7.9, 60 mM imidazole, 500 mM NaCl).  
C-terminal or N-terminal histidin tagged CuZnSOD was eluted  
three times with 1,2 ml buffer C (10 mM Tris-HCl, 500 mM  
imidazole, 250 mM NaCl). Purity and correct expression  
products were checked by immunoblotting or N-terminal protein  
30 sequencing after separation of 20 µl eluate in SDS-PAGE  
(discontinuous 12,5 % SDS-PAGE). To examine the protein  
levels in bacterial culture all CuZnSOD variants were induced  
synchronously at OD<sub>600</sub> = 0.6 with 1 mM IPTG. After 1 h, 2 h,  
3 h, 4 h and 20 h, aliquots (1 ml) of E.coli cultures were

taken, centrifuged and homogenized in buffer A as described. The pellet was resuspended in 1 ml H<sub>2</sub>O. Subsequently, 20 µl of the supernatant (soluble fractions) or 20 µl of the sonicated pellet suspension (insoluble fractions) were mixed with 7 µl of denaturing sample buffer (10 % SDS, 10 % mercaptoethanol, 20 % glycerol, 130 mM Tris-HCl pH 6.8, 0.03 % bromphenol blue). The samples were heated for 2 minutes at 80 °C and analyzed by 12 % SDS-PAGE. After coomassie staining, the electropherograms were digitized with a CCD camera (Gel Doc 1000, BioRAD) and analyzed by densitometry using NIH-Image software (1.61).

**6. Processing of CuZnSOD** - In order to remove the nonphysiological histidin tag and to yield CuZnSOD useful for clinical applications the N-terminal histidin tagged CuZnSOD was proteolytically processed with cathepsin-C or the C-terminal variant was processed with carboxypeptidase-A according to the manufacturer's protocols (Boehringer-Mannheim, Mannheim, Germany). Treatment with cathepsin-C yielded a processed CuZnSOD starting with the amino acids NH<sub>2</sub>-GSAT KAVCVLKGDGP (indicated in sequence protocol CuZnSOD-pQE30 SEQ ID NO 13). C-terminal fusion protein was yielded the C-terminal amino acid sequence VIGIAQR-COOH (indicated in sequence protocol CuZnSOD-pQE30 SEQ ID NO 13). Verification was done by peptide sequencing.

**7. Reactivation of CuZnSOD** - In order to yield physiologically relevant active homodimeric CuZnSOD, the CuZn-NTA eluate was ultrafiltered through a 5 kD membrane (Omegacell, Filtron, Northborough, MA, USA). For buffer exchange the samples were washed three times in reconstitution buffer (50 mM sodium citrate pH 5.5, 1 mM DTT). The protein solutions were incubated at 8 °C for 7 days (250 µg/ml protein). After distinct time intervals aliquots of the refolding mixture

were either analyzed by native gel electrophoresis (2.6  $\mu$ g CuZnSOD) and activity staining or assayed in a spectrophotometer (0.5 - 1  $\mu$ g CuZnSOD, superoxide dismutase assay kit, Calbiochem, San Diego, CA, USA). For visualization of protein bands native gels were stained with coomassie blue. For the production of larger CuZnSOD amounts M15-E.coli cells were subsequently grown in 15 ml, 200 ml, 2500 ml and 20 L flasks. Refolded CuZnSOD proteins were dialyzed against 100 volumes of buffer D (10 mM Tris-HCl 0.1 % Saccharose) and lyophilized.

**8. SOD assay and activity staining** - Enzymatic activity of the CuZnSOD proteins were either analyzed by 10 % native gel electrophoresis and activity staining with nitrotetrazolium blue dye or by a quantitative spectrophotometrically assay according to published protocols (Beauchamp and Fridovich, 1971; Nebot et al., 1993). Protein yields were determined by the Bradford method (Protein assay kit, BioRAD, Hercules, CA, USA). The concentration of purified CuZnSOD was determined spectrophotometrically using the extinction coefficient  $\epsilon_{265} = 1.84 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ .

**9. Subcloning of human CuZnSOD into pEGFP eukaryotic expression vector and generation of stable transfected PC12 cells (C-terminal fusion protein with enhanced green fluorescent protein as a fluorescent marker/label)** - Using 10  $\mu$ M of the primers SOD-pEGFP-s 5'-ccg cgg gcc cgc cat ggc gac gaa ggc cgt gtg cgt gc-3' and SOD-pEGFP-as 5'-gct cac cat ggt ggt ttg ggc gat ccc aat tac acc ac-3', 10 ng CuZnSOD-pQE60 vector, 300  $\mu$ M dNTPs, 5  $\mu$ l of the manufacturer's PCR buffer and 2,5 U Taq-polymerase with 25 cycles of 1 min 95  $^{\circ}$ C, 1 min 60  $^{\circ}$ C, 1 min 72 $^{\circ}$ C (50  $\mu$ l total volume, Taq-polymerase, Pharmacia Biotech, Uppsala, Sweden) a PCR product was generated which was cleaved by ApaI/NcoI digestion. The purified



PCR product was ligated into ApaI/NcoI treated pEGFP-N3 vector (Clontech Laboratories, Palo Alto, CA, USA). After amplification in XL2-Blue cells (25 µg/ ml kanamycin) and plasmid purification the CuZnSOD-pEGFP vector construct was transfected into PC12 rat adrenal pheochromocytoma cells using the CalPhos<sup>TM</sup> Transfection Kit according to the manufacturer's protocols (Clontech Laboratories, Palo Alto, CA, USA). Stable transfected CuZnSOD-pEGFP clones were selected by fluorescence microscopy (excitation 488 nm/ emission 520 nm, MRC 1024 confocal microscope, BioRAD Laboratories, Hercules, CA, USA).

#### 10. Cloning of the regulatory subunit human calcineurin-B -

Cloning of human calcineurin-B was performed by reverse transcription PCR using human brain poly-A-RNA as template (Clontech, Palo Alto, CA, USA). The oligonucleotides CNBa-s1 5'-ccg ccg acc cgc cga gca-3', CNBa-as1 5'-ggg act ctc tga taa gag-3', CNBa-s3 5'-gga att ccc cgg gga aag agg aga aat taa cta tgg gaa atg agg caa gtt atc-3', CNBa-as2 5'-ttc cgg gcc caa gct tct aat taa tca cac atc tac cac cat c-3' were supplied by Interactiva (Ulm, Germany). Reverse transcription was performed with the CNBa-as1 primer and 100 ng of poly-A-RNA according to the manufacturer's protocol (Expand reverse transcriptase, Boehringer Mannheim, Germany). The human calcineurin-B cDNA was amplified by nested PCR. The first PCR was performed in 20 µl, using 0.5 µl reverse transcription product, 10 µM CNBa-s and CNBa-as1 primers, 300 µM dNTPs, 2 µl of the manufacturer's 10 x PCR buffer and 2.5 U Pfu-polymerase with 20 cycles of 1 min 95 °C, 1 min 55 °C, 2 min 72 °C followed by a second PCR (50 µl) with 5 µl of the purified first PCR product, 10 µM CNBa-s3 and CNBa-as2 primers, 300 µM dNTPs, 5 µl of the manufacturer's PCR buffer and 2.5 U Pfu-polymerase with 20 cycles of 1 min 95 °C, 1 min

55 °C, 1 min 72 °C (Pfu-polymerase, Stratagene, San Diego, CA, USA).

#### 11. Cloning of the catalytic subunit human calcineu-

5 **rin-A-Alpha and splicevariants** - Cloning of human calcineu-  
rin-A-alpha was performed by reverse transcription PCR using  
human brain poly-A-RNA as template (Clontech, Palo Alto, CA,  
USA). The oligonucleotides CNAa-s1 5'-gcg tcg ctg tcc tcc ggc  
agc-3', CNAa-as1 5'-gtg aac agg aag tgg tca ctg-3', CNAa-s2  
10 5'-cat gcc atg gatc cat gtc cga gcc caa ggc-3', CNAa-as4  
5'-tcc ccc cgg ggta ccc tag tta atc act gaa tat tgc tgc tat  
tac-3' were supplied by Interactiva (Ulm, Germany). Reverse  
transcription was performed with the CNAa-as1 primer and 100  
ng of poly-A-RNA according to the manufacturer's protocol  
15 (Expand reverse transcriptase, Boehringer Mannheim, Germany).  
The human calcineurin-A-Alpha cDNA was amplified by nested  
PCR. The first PCR was performed in 25 µl, using 0,5 µl  
reverse transcription product, 10 µM CNAa-s1 and CNAa-as1  
primers, 200 µM dNTPs, 2.5 µl of the manufacturer's 10 x PCR  
20 buffer and 1.25 U Pfu-polymerase with 30 cycles of 40 seconds  
at 95 °C, 40 seconds at 55 °C, 3 min 72 °C followed by a  
second PCR (25 µl) with 2.5 µl of the purified first PCR  
product, 10 µM CNAa-s2 and CNAa-as2 primers, 200 µM dNTPs,  
2.5 µl of the manufacturer's PCR buffer and 2.5 U Pfu-poly-  
25 merase with 25 cycles of 40 seconds at 95 °C, 40 seconds at  
55 °C, 3 min 72 °C (Pfu-polymerase, Stratagene, San Diego,  
CA, USA).

Hereby a new splicevariant was identified, which is impor-  
30 tant for calcium regulation and proteolytic regulation of  
calcineurin-A. The splicevariant lacks the hole catalytic  
phosphatase domain and part of calcineurin-binding-site  
(Elimination of nucleic bases 208-1317 in sequence protocol

CNAa1-pQE30 SEQ ID NO 17). The corresponding vector is named CNAa3-pQE30:

#### Location/Qualifiers

- 5 151..606 /note="splicevariant: Calcineurin A alpha 1 lacking phosphatase domain, newly generated N-terminus exhibits protease activity"  
 115..150 /note="His-Tag"  
 649..1161 /note="Calcineurin B;Calcineurin B alpha Ca<sup>2+</sup>  
 10 binding"

**12. Cloning of the catalytic subunit human calcineurin-A-Beta and splicevariants** - PCR was performed as described under 11. with the exception that the primers CNAb-s1 5'-gag cct agc  
 15 cga gcc ccg gg-3' and CNAb-as1 5'-ctg gga agt agt ggg tca ctg-3' were used for the first PCR and the primers and CNAb-s2 5'- cat gcc atg gat cca tgg ccg ccc cgg agc c-3' and CNAb-as4 5'- tcc ccc cgg ggt acc cta gtt aat cac tgg gca gta tgg ttg cca g-3' were used for second PCR.

**13. Cloning of the catalytic subunit human calcineurin-A-Gamma and splicevariants** - PCR was performed as described under 11. with the exception that the primers CNAg-s1 5'-gga gcc  
 25 tgg agg agg ccg ag-3' and CNAg-as1 5'-cgg cag gac tct aag tca tga-3' were used for the first PCR and the primers and CNAg-s2 5'-cat gcc atg gat cca tgt ccg gga ggc gct tc-3' and CNAg-as4 5'-tcc ccc cgg ggt acc cta gtt aat cat gaa tgg gct ttc ttc cct t-3' were used for second PCR.

- 30 Hereby a new splicevariant was identified, which is important for calcium regulation and proteolytic regulation of calcineurin-A. The splicevariant with human exon is not yet available in gene database (substitution of nucleic bases 1474-1503 in sequence protocol CNAg2-pQE30 SEQ ID NO 32) with

5'-ACA GTA GAA GCG GTA GAG GCC CGG GAA GCC-3' (corresponding peptide: NH<sub>2</sub>-TVEAVEAREA-COOH). The corresponding vector is named CNAg3-pQE30.

#### 5 Location/Qualifiers

115..150 /note="His-Tag"  
 151..1689 /note="Calcineurin-A-Gamma-2"  
 1474..1503 /note="human brain calcineurin-A-gamma alternative exon = interaction domain with cytoskeleton, death-domain homolog, stomatin homolog"  
 1690..1731 /note="RBS&MCS2"  
 1732..2244 /note="Calcineurin-B"

#### 14. Subcloning of calcineurin-B and calcineurin-A variants

- 15 into pQE30 - For the recombinant expression in procaryotic cells calcineurin-B was subcloned with either calcineurin-A-alpha1, calcineurin-A-alpha2, calcineurin-A-beta1, calcineurin-A-beta2, calcineurin-A-gamma1 or calcineurin-A-gamma2. The purified calcineurin-A-alpha, calcineurin-B-alpha or calcineurin-A-gamma PCR products (described in 11. - 13.) were restricted with BamHI/XmaI. The purified calcineurin-B product (described in 10.) was restricted with XmaI/HindIII and ligated together with the respective calcineurin-A-fragment into the BamHI/HindIII treated vector
- 25 pQE30 to yield the final procaryotic expression vector constructs CNAa1-pQE30, CNAa2-pQE30, CNAa3-pQE30, CNAb1-pQE30, CNAb2-pQE30, CNAg1-pQE30, CNAg2-pQE30 and CNAg3-pQE30.

#### 15. Recombinant coexpression and purification of calcineu-

- 30 rin-B/calcineurin-A heterodimers with CuZnSOD - CNAa1-pQE30, CNAa2-pQE30, CNAa3-pQE30, CNAb1-pQE30, CNAb2-pQE30, CNAg1-pQE30, CNAg2-pQE30 or CNAg3-pQE30 were transformed into E.coli M15[pREP4][CuZnSOD-pQE30] to yield cells able to coexpress calcineurin-A, calcineurin-B and CuZnSOD. cells

were plated on LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) agar. Expression cultures were grown in 250 ml LB / ampicillin (100 µg/ml) / kanamycin (25 µg/ml) until the OD<sub>600</sub> was 0.6. Constitutive leakage expression was prevented by the repressor plasmid pREP4-lacI. Production of the human calcineurin-A/calcineurin-B histidin tagged heterodimers was induced by addition of IPTG (1 mM). After four hours the bacterial cells were harvested by centrifugation (4000 g, 20 min), resuspended in 8 ml buffer A (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl) and homogenized by three freeze thaw cycles and sonication on ice (Bandelin sonoplus GM70, 300 W, 3 x 10 sec). The lysate was centrifuged (10,000 g, 20 min) and incubated with 750 µl Fe-NTA-agarose for batch affinity binding for 1 h at 4 °C (Qiagen expressionist kit, Qiagen, Hilden, Germany). Fe-NTA-agarose was prepared from Ni-NTA-agarose (Qiagen expressionist kit, Qiagen, Hilden, Germany) by subsequent washes in:

- 1) 2 volumes of bidestilled water
- 2) 3 volumes of regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)
- 3) 5 volumes bidestilled water
- 4) 3 volumes 2% SDS
- 5) 1 volume 25% ethanol
- 6) 1 volume 50% ethanol
- 7) 1 volume 75% ethanol
- 8) 5 volumes 100% ethanol
- 9) 1 volume 75% ethanol
- 10) 1 volume 50% ethanol
- 11) 1 volume 25% ethanol
- 12) 1 volume bidestilled water
- 13) 5 volumes 100 mM Na-EDTA pH 8.0
- 14) 5 volumes bidestilled water
- 15) 2 volumes 100 mM FeSO<sub>4</sub> / 1 mM reduced glutathion / 1 mM dithiothreitol/ 100 mM ascorbic acid

- 16) 2 volumes bidestilled water  
17) 2 volumes regeneration buffer (6 M guanidiniumhydrochloride, 0.2 M acetic acid)  
18) 2 volumes buffer buffer A3 (20 mM Tris-HCl pH 7.9, 5 mM  
5 imidazole, 500 mM NaCl, 200  $\mu$ M FeSO<sub>4</sub> / 1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid

The batch was applied to a 30 ml chromatography column, washed with 15 ml buffer A4 (20 mM Tris-HCl pH 7.9, 5 mM  
10 imidazole, 500 mM NaCl/ 1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid) and subsequently with 8 ml buffer B (20 mM Tris-HCl pH 7.9, 60 mM imidazole, 500 mM NaCl/ 1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid). N-terminal histidin tagged calcineurin-A/cal-  
15 cineurin-B heterodimer was eluted three times with 1.2 ml buffer C (10 mM Tris-HCl, 500 mM imidazole, 250 mM NaCl/1 mM reduced glutathion / 1 mM dithiothreitol/ 1 mM ascorbic acid, buffer was degased and subsequently saturated with nitrogen). To prevent oxidation of calcineurin, the eluate  
20 was stored at -80 °C in nitrogen containing and oxygen free vials. Purity and correct expression products were checked by immunoblotting or N-terminal protein sequencing after separation of 20  $\mu$ l eluate in SDS-PAGE (discontinuous 12,5 % SDS-PAGE).

25

- 16. Subcloning of human calcineurin-A-Alpha into pEGFP eukaryotic expression vector and generation of stable transfected PC12 cells (C-terminal fusion protein with enhanced green fluorescent protein as a fluorescent marker) -** The  
30 vector CNAA2-pQE30 was digested with BamHI/XmaI to generate a sticky end CNAA2 fragment. The purified fragment was ligated into Bgl-II/XmaI treated pEGFP-C1 vector (Clontech Laboratories, Palo Alto, CA, USA). After amplification in XL2-Blue cells (25  $\mu$ g/ ml kanamycin) and plasmid purification the

CNAa-pEGFP vector construct was transfected into PC12 rat adrenal pheochromocytoma cells using the CalPhos<sup>TM</sup> Transfection Kit according to the manufacturer's protocols (Clontech Laboratories, Palo Alto, CA, USA). Stable transfected CNAa-pEGFP clones were selected by fluorescence microscopy during a three month propagation procedure (excitation 488 nm/emission 520 nm, MRC 1024 confocal microscope, BioRAD Laboratories, Hercules, CA, USA).

10 **17. Subcloning of calcineurin-A-Beta into pEGFP** - The same procedure as described in 16. was applied except that the CNAa2-pQE30 vector was substituted by CNAb2-pQE30 to generate CNAb-pEGFP.

15 **18. Subcloning of calcineurin-A-Gamma into pEGFP** - The same procedure as described in 16. was applied except that the CNAa2-pQE30 vector was substituted by CNAg2-pQE30 to generate CNAg-pEGFP.

20 **19. Western blotting and protein sequencing** - Transfer of purified proteins from 12% SDS-PAGE to PVDF membranes (Boehringer-Mannheim, Mannheim, Germany) was performed according to standard protocols using transfer buffer (48 mM Tris, 39mM Glycine, 20% methanol, 1% SDS, pH 9.2) and following blotting  
25 conditions: 75 min at 25 V/110 mA. Blocking, washing and detection (HRP detection system) were performed according to the manufacturer's protocols (ECL kit, Amersham, Buckinghamshire, UK). An anti-human CuZnSOD antibody (1:5,000 dilution, rabbit polyclonal anti-human SOD1 antibody; BIOMOL, Hamburg,  
30 Germany) was used as primary antibody and an anti-rabbit IgG antibody (1:10,000 dilution) labeled with HRP was used as secondary antibody. For the detection of calcineurin-A (alpha, beta, gamma isoforms) a polyclonal calcineurin-A antibody was used as 1:5000 dilution (Sigma Aldrich, Deisen-

hofen, Germany). For N-terminal protein sequencing the PVDF membrane was soaked in 100% methanol. Proteins which seemed to be blocked by N-terminal posttranslational modifications were treated with acylamino-acid-peptidase according to the manufacturer's protocol (Boehringer-Mannheim, Mannheim, Germany). Coomassie brilliant blue stained bands were cut out. Automated Edman degradation of peptides was performed on an Applied Biosystems protein sequencer (476A).

20. **Calcineurin phosphatase assay** - 100 ng - 4  $\mu$ g recombinant calcineurin (calcineurin-A/B heterodimer), 100 ng - 1  $\mu$ g purified bovine brain calcineurin (Sigma Aldrich, Deisenhofen, Germany) or 100  $\mu$ g homogenized tissue or cell extracts were used for classical calcineurin phosphatase assays. 100  $\mu$ g cells or tissue were homogenized exactly as described (Stemmer et al., 1995). Partly purified and redox sensitive calcineurin was prepared by centrifugation at 14,000 rpm at 4°C for 10 min (Eppendorf Centrifuge 5417R) and the resulting supernatant was separated on a 1.5 x 10 cm Sephadex-G50 gelfiltration column as described (Stemmer et al., 1995), (Gold et al., 1997). Phosphotyrosine phosphatase assay was performed in microplates (100  $\mu$ l total assay volume) either using 30  $\mu$ M fluoresceinmonophosphate or 20 mM para-nitrophenylphosphate (Sigma Aldrich, Deisenhofen, Germany); 10  $\mu$ l recombinant, purified or partly purified and assay buffer (25 mM Tris/HCl, pH 7; 2 mM  $\text{CaCl}_2$ ; 0.1  $\mu$ M calmodulin; 25  $\mu$ M FK506). After starting the enzymatic reaction with para-nitrophenylphosphate or fluoresceinmonophosphate the absorbance at 405 nm (para-nitrophenylphosphate) or fluorescence ( $\lambda$  excitation = 485 nm;  $\lambda$  emission = 520 nm) was monitored over 20 min at 30°C using a UV/VIS/fluorescence microplate photometer (Biolumin 960 kinetic fluorescence/absorbance photometer, Molecular Dynamics). Phosphoserine phosphatase assay was performed as described (Hubbard and Klee, 1991), (Wang et



al., 1996). In short: 40  $\mu$ l recombinant or partly purified calcineurin was mixed with test buffer (40 mM Tris/HCl pH 8; 0.1 M KCl; 0.4 mg/ml BSA; 0.67 mM DTT; 0.67  $\mu$ M calmodulin; 1  $\mu$ M FKBP binding protein; 0.5  $\mu$ M ocadaic acid for inhibition of phosphatase A1 and A2) and enzymatic reaction and calcium induced redox-inactivation of calcineurin started by addition of 20  $\mu$ l substrate buffer (7.7  $\mu$ M radioactive phosphorylated RII-peptid, 2.0 mM  $\text{CaCl}_2$ ). The assay was performed in duplicates and the addition of 1  $\mu$ M FK506 or cyclosporine was used to verify calcineurin activity for each reading point. The protective effect of CuZnSOD against redox inactivation of calcineurin was determined by addition of 3  $\mu$ g recombinant human wild-type or mutated CuZnSOD (constant CuZnSOD protein) or addition of 1.67 units of recombinant human wild-type or mutated CuZnSOD (constant CuZnSOD activity). The reaction mixture was incubated for 2 min at 30°C and stopped with 100 mM potassium phosphate / 5% TCA. The reaction mixture was passed through a 0.5 ml ion-exchange column (Dowex; AG 50W-X8, BioRad) and the unbound phosphate eluted with 0.5 ml water. The quantity of released phosphate was determined by a scintillation counting.

An enzymatic protein phosphatase assay was established using the nonphysiological substrate fluoresceinmonophosphate (FMP). Assuming a Michaelis-Menten kinetic for FMP and using the Lineweaver-Burk method for analysis of kinetic data a  $K_M$  of 40  $\mu$ M and a  $V_{\max}$  of 400  $\mu$ mol / min was determined. The assay was applicable to calcineurin and magnesium dependent proteinphosphatase 2C (data not shown, (Grothe et al., 1998)). The enzymatic activity is linear in the range of 12.5 pM to 75 pM calcineurin. FMP is more sensitive than para-nitrophenylphosphate (pNPP). Neither FMP nor pNPP are useful to measure calcineurin activity in crude preparations by inhibition with the immunosuppressive drugs FK506 or cyclosporine

(cell homogenate, partly purified calcineurin). Both substrates also failed to measure calcium induced redox-inactivation of calcineurin or CuZnSOD mediated protection of this inactivation. The inhibition assay also failed when calcium was substituted against other divalent cations ( $\text{Ni}^{2+}$ ,  $\text{Mg}^{2+}$ ). Only the physiological relevant substrate could be used in an immunosuppressive drug inhibitory assay (RII-peptide phosphopeptide). In the classic radioactive assay 95% inhibition with 1  $\mu\text{M}$  FK506 or cyclosporine was determined. It is concluded that inhibition of calcineurin activity by immunosuppressive drugs needs larger molecular weight substrates than pNPP and FMP. Furthermore it is concluded that redox-sensitivity is linked to phosphoserine phosphatase activity and therefore not detectable with phosphotyrosine analoges like pNPP or FMP. The recombinant human wild-type CuZnSOD and purified human erythrocyte CuZnSOD (Sigma Aldrich, Deisenhofen, Germany) were effective to protect 50-100% of calcineurin after calcium induced redox inactivation. Mutated CuZnSOD proteins, associated with the severe neurological disorder amyotrophic lateral sclerosis, were less effective to protect calcineurin against redox inactivation.

#### Protective effect of CuZnSOD of calcium induced inactivation of calcineurin

25

Percentage of FK506 inhibitable RII-phosphopeptide activity after 20 min compared with the activity at 0 min

human CuZnSOD	constant protein (3 $\mu\text{g}$ )	constant activity (1.67 U)
erythrocyte wild-type (8330 U/mg)	57 +/-10 %	57 +/-10 %
recombinant wild-type	70 +/-33 %	58 +/-22 %

(6380 U / mg)

recomb. mutation D90A	42 +/-17 %	32 +/-15 %
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(4590 U/ mg)

recomb. mutation G93A	16 +/-16 %	21 +/-22 %
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5 (2130 U/ mg)

recomb. mutation A4V	22 +/-27 %	8 +/-3 %
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(1820 U/ mg)

control (no CuZnSOD)	9 +/-7 %	9 +/-7 %
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(0 U / mg)

10 The protective effect does not depend on CuZnSOD activity since higher protein amounts of mutated CuZnSOD corresponding to a higher enzymatic activity were even less effective in protection of calcineurin.

15 Therefore it is concluded that amino acid substitutions, associated with familial amyotrophic lateral sclerosis, are important for the protein interaction of calcineurin and CuZnSOD and therefore are involved in the CuZnSOD mediated  
20 protection of calcium induced redox inactivation of calcineurin. Since this protective effect is disturbed in amyotrophic lateral sclerosis and protection of calcineurin by CuZnSOD it may also be important in other neurological and cardiovascular diseases (Alzheimer, Parkinson, epilepsy, ischemia,  
25 heart-failure).

An high-throughput bioassay was developed to detect and isolate artificial or endogenous drugs enhancing (activators) CuZnSOD-calcineurin interaction and therefore protecting  
30 calcineurin against redox-inactivation or drugs reducing (inhibitors) CuZnSOD-calcineurin interaction and therefore inhibit calcineurin activity. Inhibitors are useful to substitute toxic immunosuppressive drugs like FK506 or cyclosporine. Activators and inhibitors may be useful for the

therapeutical treatment of amyotrophic lateral sclerosis, Parkinson, Alzheimer, epilepsy, ischemia and cardiovascular diseases.

21. High Throughput BioAssay using recombinant calcineurin-A, recombinant calcineurin-B, calmodulin and recombinant CuZnSOD (analytical assay to identify activators or inhibitors of CuZnSOD/calcineurin interaction) - Laser fluctuation correlation spectroscopy (FCS) is a useful tool to quantify ligand-ligand interactions. The fluorescence  $F(t)$  of a optical well defined volume element which is excited by a confocal laser is monitored as a function of time. The temporal autocorrelation of the fluorescence fluctuation  $\delta F(t)$  yields the time scale of this dynamics and the average number of independent fluorophores in the probe volume. If the fluorescence fluctuation arise from diffusive motion and from fluorescence sensitive reaction, fluorescent fluctuation correlation function signal is approximated by the formula:

$$G_{DR}(\tau) = G_{Diff}(\tau) * [1 + A * \exp(-k_R * \tau)]$$

$\tau$  = fluorescence correlation time

$k_R$  = apparent binding constant of the fluorescent labeled ligand

$A$  = equilibrium coefficient dependent constant

If one measurement is performed with a solution only containing the fluorescent labeled ligand and a second measurement is performed with a solution containing the fluorescent labeled ligand and an interacting molecule the correlation function  $G_{DR}$  can be separately analyzed and yields binding parameters of the interacting molecules. Upon binding of the ligand to the interacting molecule the hydrodynamic radius increases and therefore the diffusion coefficient decreases resulting in a longer correlation time.

A fluorescence labeled recombinant CuZnSOD as a fluorescent label was used to monitor the binding dynamics to calcineurin. CuZnSOD was labeled with Oregon-Green-514 dye according to the manufacturer's protocols (FluoReporter Protein labeling Kit, Molecular Probes, Leiden, Netherlands). The amount of fluorescent dye labels per CuZnSOD dimer was quantified by determining the ratio of the absorbance at 265 nm (CuZnSOD protein) / 514 nm (Oregon-dye). The diffusion constant and correlation time of the labeled CuZnSOD (100 nM) was measured on a bovine serum albumin treated glass plate with a confocal laser microscope attached to an autocorrelator ( $\lambda$  excitation = 488 nm,  $\lambda$  emission = 511 nm) in 10  $\mu$ l assay buffer containing 50 mM sodiumphosphate pH 7.1, 150 mM NaCl, 0.67 mM DTT, 0.67  $\mu$ M calmodulin, 0.67 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ . The beam from a modelocked Ti:Sa or cw argon ionlaser was collimated to fill the back aperture of a immersion microscope objective (Zeiss C-Apochromat 63x1.2w), producing a small diffractionlimited spot. The emitting fluorescent light was collected by the same objective separated from the excitation light by a beamsplitter/filter combination and imaged first to a variable pinhole and than to the detector (Avalanche Photodiode EG&G SPCM AQ161 or PMT Hamamatsu R5600-03). The labeled CuZnSOD exhibited an autocorrelation time corresponding to a hydrodynamic radius of 41,000 Dalton which is comparable to the expected molecular weight of the homodimer (34,600 Dalton). Next 0.2  $\mu$ l calcineurin-A/B heterodimer (5  $\mu$ M) was added to a labeled CuZnSOD mixture and the fluorescence correlation signal was determined. The hydrodynamic radius increases from 41 kDa to 90 kDa indicating that approximatly one calcineurin heterodimer interacts with one CuZnSOD dimer (expected: 114 kDa). Using mutated D90A CuZnSOD yielded an apparent molecular weight of 180.000 kDa indicating the formation of calcineurin/CuZnSOD aggrega-

tes. The apparent binding constant between human wild-type CuZnSOD and calcineurin was estimated as  $k_D = 2 \times 10^{-6} \text{ M} \pm 1 \times 10^{-6} \text{ M}$ . It is concluded that laser correlation spectroscopy is useful to perform a ultra high throughput screening for ligands diminishing the CuZnSOD/calcineurin interaction which simply can be monitored by a reduction of the autocorrelation time after addition of a potential drugs. It is possible to screen for suitable substances using substances available in chemical, peptide or natural compound screening libraries.

**22. High Throughput BioAssay using recombinant calcineurin-A, recombinant calcineurin-B, calmodulin and RII-Fluophos (analytical assay to identify activators or inhibitors of**

**calcineurin)** - RII peptide was synthesized according to standard peptide synthesis protocols ((Blumenthal et al., 1988); Interactiva, Ulm, Germany). To generate a fluorescent labeled peptide which furthermore contains a phosphoester at Ser-15, amino acid residue Ser-15 was coupled with fluoresceine-phosphoamidit (FluoreDite Labeling Reagent, Perseptive Biosystems), which is usually used for labeling of nucleotides, to yield RII-Fluophos (Interactiva, Ulm, Germany). The expected molecular weight (2578.8 Dalton) was confirmed by mass spectrometry (2580.6 Dalton). The Fluophos-RII-peptide was not converted by calcineurin as was monitored by fluorescence spectrometrie (Biolumin 960 UV-VIS/fluorescence microplate reader). Therefore Fluophos-RII-peptide was used in laser fluorescence correlation spectroscopy as described in paragraph 20 except that  $\lambda$  excitation was 488 nm and  $\lambda$  emission was 520 nm. Furthermore, labeled CuZnSOD was substituted by 10 nM Fluophos-RII-peptide yielding a hydrodynamic radius corresponding to 4 kDa (expected 2.6 kDa). After calcineurin addition the molecular weight increases to 100.000 kDa and a binding

constant of  $K_d = 0.6 \times 10^{-6}$  M is estimated. Binding constants were comparable between the six calcineurin isoforms/splicevariants. It is concluded that laser correlation spectroscopy is useful to perform a ultra high throughput screening for ligands directly substrate binding to calcineurin by simply monitoring the autocorrelation time after addition of potential drugs. By descriminating the binding properties of potential drugs to the six different heterodimer combinations (calcineurin-A-alpha1/calcineurin-B, calcineurin-A-alpha2/calcineurin-B, calcineurin-A-beta1/calcineurin-B, calcineurin-A-beta2/calcineurin-B, calcineurin-A-gamma1/calcineurin-B, calcineurin-A-gamma2/calcineurin-B) it is possible to identify tissue specific and therefore less toxic calcineurin inhibitors.

It is possible to combine the screening procedures described in paragraph 20 and 21 strategically: substances which are able to inhibit the calcineurin-CuZnSOD interaction (positive hit in paragraph 20) but failed to show an effect in procedure 21 (negative hit) are predominantly positive candidates for the therapeutical use in neurological disorders because a toxic immunosuppressive side effect is less probable. Substances which fail to inhibit the calcineurin-CuZnSOD interaction (negative hit) but show an effect in procedure 21 (positive hit) are predominantly positive candidates for immunosuppression. Substances effective in both procedures are likely to be toxic.

**23. Cellular BioAssay using eucaryotic cells transfected with calcineurin-A-EGFP fusionprotein or CuZnSOD-EGFP fusion protein** - PC12 cells stably transfected with CuZnSOD and calcineurin isoenzymes serve as a model for monitoring the effects of CuZnSOD or calcineurin overexpression in neuronal cells. CuZnSOD reportedly has been implicated to be involved

in the mediation of hypoxie tolerance, whereas calcineurin overexpression is associated with epileptogenesis, Parkinsonism or Alzheimers disease. It is possible to use theses cells subsequently to the identification of potential drugs in screening protocols 20 und 21. Toxicity of potential neuroprotective drugs and the effect on the subcellular distribution of calcineurin-isoforms or CuZnSOD, respectively can be monitored.

24. Pull-Down-BioAssay using histidine tagged recombinant CuZnSOD to purify CuZnSOD interacting ligands (Preparative assay to isolate activators or inhibitors of CuZnSOD/calcineurin interaction from biological sources) - Recombinant purified histidine tagged CuZnSOD in 50 mM sodiumphosphate buffer pH 8.0 was attached to CuZn-NTA magnetic agarose beads by incubating 100  $\mu$ l beads suspension with 100  $\mu$ l CuZnSOD solution (0.3  $\mu$ g/ $\mu$ l) in 96 well microplates for 30 minutes at room temperature on a microplate shaker (600 rpm). CuZn-NTA magnetic beads were generated from Ni-NTA beads by applying the same procedure as described under paragraph 5 (Ni-NTA magnetic agarose beads, Qiagen, Hilden, Germany). The microplate was placed on the 96 well magnet for 1 minute and the supernatant removed from the wells.
- Cytosolic ligands were isolated as follows: 200  $\mu$ l interaction buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 20 mM imidazole pH 8.0, 0.1 % Tween-80) were added to the CuZn-NTA agarose beads/CuZnSOD containig wells and placed on the 96 well magnet to remove interaction buffer. 100 mg tissue, cells or other biological specimen to be analyzed for CuZnSOD interacting ligands were homogenized in 200  $\mu$ l lysis buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 10 mM imidazole pH 8.0, 0.1 % Tween-80) using a dounce homogenizer. The lysate was cleared by 30 min centrifugation at 10,000 g at 4 °C. The supernatant was



applied to the wells containing CuZn-NTA absorbed recombinant human CuZnSOD, mixed and incubated for 60 minutes at 0 °C. The microplate was placed on the 96 well magnet for 1 minute to remove the supernatant. After removal of the lysate the wells were washed twice by adding 200 µl interaction buffer. Elution of CuZnSOD and interacting ligands was achieved by addition of 100 µl elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole pH 8.0, 0.1 % Tween-80).

- 10 Membranous ligands were isolated as follows: denaturing interaction buffer (6 M guanidine-HCL, 100 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 0.1 % Tween-80) was added to the CuZn-NTA agarose beads/ CuZnSOD containig wells and placed on the 96 well magnet to remove interaction buffer. The pellet from the procedure
- 15 above was solubilized in 200 µl denaturing interaction buffer (6 M guanidine-HCL, 100 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 0.1 % Tween-80) for 60 minutes at room temperature. The solubilisate was cleared by 30 min centrifugation at 10,000 g at room temperature. The supernatant was applied to the wells containing
- 20 CuZn-NTA absorbed recombinant human CuZnSOD, mixed and incubated for 60 minutes at room temperature. The microplate was placed on the 96 well magnet for 1 minute to remove the supernatant. The wells were washed once with 200 µl denaturing interaction buffer (6 M guanidine-HCL, 100 mM NaH<sub>2</sub>PO<sub>4</sub> pH
- 25 8.0, 0.1 % Tween-80) and a second time with 200 µl denaturing wash buffer (8 M Urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0, 0.1 % Tween-80). Elution of CuZnSOD and interacting ligands was achieved by addition of 100 µl denaturing elution buffer (8 M Urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub> pH 4.0, 0.1 % Tween-80).

30

To remove low molecular weight ligands for HPLC analysis, the eluates (cytosolic or membranous) were ultrafiltered through a 5 kDa membrane as described under 7. Low molecular weight ligands were separated on a preparative reverse phase HPLC

(UV detection at 200 nm). Homogeneity and molecular weight of UV detectable fractions were analyzed by mass spectrometry. High molecular weight ligands (ultrafiltration remainder) were separated on a 10% polyacrylamide gel and protein bands identified by sequencing or MALDI mass spectrometrie as described under 19. Interacting nucleic acid was analyzed by separating the membranous eluate on a 1 % agarose gel and staining with ethidium bromide. Fluorescent bands were extracted from the agarose (Qiagen gel extraktion kit, Qiagen, Hilden, Germany) subjected to digestion with RsaI and subcloned into RsaI treated pQE30 vector for DNA sequencing.

**25. Pull-Down-BioAssay using histidine tagged recombinant calcineurin-A and calcineurin-B to purify calcineurin interacting ligands (Preparative assay to isolate activators or inhibitors of CuZnSOD/calcineurin interaction from biological sources) -** Isolation and identification of calcineurin interacting ligands was performed analogous to paragraph 24 with the exception that recombinant calcineurin-A/B heterodimer was attached to Fe-NTA magnetic agarose beads which were prepared as described under paragraph 15. Furthermore six different heterodimer combinations were used (calcineurin-A-alpha1/calcineurin-B, calcineurin-A-alpha2/calcineurin-B, calcineurin-A-beta1/calcineurin-B, calcineurin-A-beta2/calcineurin-B, calcineurin-A-gamma1/calcineurin-B, calcineurin-A-gamma2/calcineurin-B) to descriminate between isoenzyme and splicevariant specific interaction partners.

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#### Content of sequence listing

- 20 1. eukaryotic expression vector CuZnSOD-EGFP (CuZnSOD-pEGFP) (DNA)
2. CuZnSOD (PRT)
3. EGFP (PRT)
4. eukaryotic expression vector EGFP-Calcineurin A alpha (CNAa-pEGFP) (DNA)
- 25 5. EGFP (PRT)
6. Calcineurin A alpha (PRT)
7. eukaryotic expression vector EGFP-Calcineurin A beta (CNAb-pEGFP) (DNA)
8. EGFP (PRT)
- 30 9. Calcineurin A beta (PRT)
10. eukaryotic expression vector EGFP-Calcineurin A gamma (CNAg-pEGFP) (DNA)
11. EGFP (PRT)
12. Calcineurin A gamma (PRT)

13. prokaryotic expression vector His-CuZnSOD (CuZnSOD-pQE30) (DNA)
14. CuZnSOD (PRT)
15. prokaryotic expression vector CuZnSOD-His (CuZnSOD-pQE60) (DNA)
- 5 16. CuZnSOD (PRT)
17. prokaryotic expression vector His-Calcineurin A alpha1-Calcineurin B (CNAa1-pQE30) (DNA)
18. Calcineurin A alpha1 (PRT)
- 10 19. Calcineurin B (PRT)
20. prokaryotic expression vector His-Calcineurin A alpha2-Calcineurin B (CNAa2-pQE30) (DNA)
21. Calcineurin A alpha2 (PRT)
22. Calcineurin B (PRT)
- 15 23. prokaryotic expression vector His-Calcineurin A beta1-Calcineurin B (CNAb1-pQE30) (DNA)
24. Calcineurin A beta1 (PRT)
25. Calcineurin B (PRT)
26. prokaryotic expression vector His-Calcineurin A beta2-Calcineurin B (CNAb2-pQE30) (DNA)
- 20 27. Calcineurin A beta2 (PRT)
28. Calcineurin B (PRT)
29. prokaryotic expression vector His-Calcineurin A gamma1-Calcineurin B (CNAg1-pQE30) (DNA)
- 25 30. Calcineurin A gamma1 (PRT)
31. Calcineurin B (PRT)
32. prokaryotic expression vector His-Calcineurin A gamma2-Calcineurin B (CNAg2-pQE30) (DNA)
33. Calcineurin A gamma2 (PRT)
- 30 34. Calcineurin B (PRT)
35. peptide RII (PRT)

ART 34 ANDT

Amended claims**Claims:**

1. Method for screening of modulators of calcineurin activity, characterized in that an interaction between calcineurin and superoxide dismutase is monitored, comprising the following steps
  - forming of a complex comprising at least calcineurin and superoxide dismutase under incubation with at least one potential modulator,
  - detecting the influence of the potential modulator by directly monitoring the complex formation and/or by monitoring the activity, especially the enzymatical activity of the complex.
2. Method according to claim 1, characterized in that the superoxide dismutase is a Copper/Zinc-superoxide dismutase.
3. Method according to claim 1 or 2, characterized in that forming of the complex is performed in the presence of the potential modulator.
4. Method according to claim 1 or 2, characterized in that the potential modulator is added after the complex has been formed.
5. Method according to one of the preceding claims, characterized in that the monitoring is performed by detection of labels, especially fluorescent labels.
6. Method according to one of the preceding claims, characterized in that calcineurin and/or superoxide dismutase

carry labels, especially fluorescent markers, wherein preferably the labels are enhanced green fluorescent protein.

7. Method according to claim 6, characterized in that calcineurin and/or superoxide dismutase are expressed as fluorescent proteins, particularly as fusion proteins together with enhanced green fluorescent protein.
8. Method according to one of the preceding claims, characterized in that the monitoring of complex formation is performed by laser fluctuation correlation spectroscopy.
9. Method according to one of the preceding claims, characterized in that calcineurin and superoxide dismutase are coexpressed in cells, especially in eukaryotic cells, and that the complex formation is performed within the cell.
10. Method according to one of the preceding claims, characterized in that calcineurin and/or superoxide dismutase are expressed in cells, especially in prokaryotic cells, and that calcineurin and/or superoxide dismutase are isolated and/or purified before the complex formation is performed.
11. Method according to claim 10, characterized in that purification of calcineurin is achieved by affinity chromatography, especially by ferro-nitrilotriacetate-metal affinity chromatography.
12. Method according to claim 10, characterized in that purification of superoxide dismutase is achieved by



affinity chromatography, especially by copper/zinc-nitrilotriacetat-metal affinity chromatography.

13. Method according to one of the preceding claims, characterized in that in the complex formation step additionally calmodulin and/or calcium is added.
14. Method according to one of the preceding claims, characterized in that the monitoring of the enzymatical activity is performed by analyzing the phosphatase activity of calcineurin.
15. Method according to claim 14, characterized in that the phosphatase activity is analyzed by the use of at least one substrate, which preferably carries a label, especially a fluorescent label.
16. Method according to claim 15, characterized in that the substrate is a peptide, especially a peptide characterized by the amino acid sequence  
Asp - Leu - Asp - Val - Pro - Ile - Pro - Gly - Arg  
- Phe - Asp - Arg - Arg - Val - Ser - Val - Ala -  
Ala - Glu.
17. Method according to claim 15 or 16, characterized in that the substrate is a peptide containing a residue, especially a serine residue, labeled with fluoresceine.
18. Method according to one of claims 3 to 17, characterized in that prior or after detecting the influence of the potential modulator on the complex formation and/or complex activity the influence of the potential modulator on the activity, especially the enzymatical activity of calcineurin is detected separately.

19. Method for screening of modulators of calcineurin activity, especially according to one of the preceding claims, comprising

- a) determining the interaction of a potential modulator with either calcineurin or superoxide dismutase as a partner,
- b) taking a potential modulator showing interaction with calcineurin or superoxide dismutase according to step a),
- c) determining the interaction of said modulator taken in step b), with the other partner, namely calcineurin or superoxide dismutase, respectively, and
- d) identifying the potential modulator showing interaction also according to step c).

20. Method according to claim 19, characterized in that calcineurin and/or superoxide dismutase comprises at least one tag, especially a histidine tag.

21. Method according to claim 19 or claim 20, characterized in that said superoxide dismutase is a Copper/Zinc-superoxide dismutase.

22. Method according to one of claims 19 to 21, characterized in that calcineurin and/or superoxide dismutase is attached to a solid matrix, especially a Ni-NTA, Fe-NTA and/or CuZn-NTA matrix.

23. Kit for screening of modulators of calcineurin activity comprising

- calcineurin and/or a vector encoding for calci-

neurin and/or cells capable of expressing calcineurin, and

- superoxide dismutase and/or a vector encoding for superoxide dismutase and/or cells capable of expressing superoxide dismutase.

- - - - -

DECLARATION FOR PATENT APPLICATION

Attorney Docket 24498

As a below-named inventor(s), I/we hereby declare that

My/Our residence(s), post office address(es) and citizenship(s) is/are as stated below next to my/our name(s)

I/We believe I/we am/are the original inventor, first and sole (if only one name is listed below) or the original, first and joint inventors (if plural names are listed below) of the subject matter which is claimed, and for which a patent is sought on the invention entitled:

**Method for screening of modulators of calcineurin activity**

the specification of which: (check one)

☐ is attached hereto.

☒ was filed on 22 July 1999, as Serial No. PCT/EP99/05220,  
19 Jan. 2001 09/744,016  
 and was amended on \_\_\_\_\_ 19 \_\_\_\_\_ (if applicable).

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the patentability of this application as defined by 37 CFR § 1.56.

We hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below, and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Applications:

<u>98 11 3876.1</u>	<u><del>XXXXXX</del> Europe</u>	<u>22 / 07 / 1998</u>	Priority Claimed
(Application No.)	(Country)	(Day/Month/Year Filed)	<input checked="" type="checkbox"/> [ ] Yes No
<u>                    </u>	<u>                    </u>	<u>      /      /      </u>	[ ] [ ] Yes No
(Application No.)	(Country)	(Day/Month/Year Filed)	
<u>                    </u>	<u>                    </u>	<u>      /      /      </u>	[ ] [ ] Yes No
(Application No.)	(Country)	(Day/Month/Year Filed)	

We hereby appoint Gary M. Nath, Reg. No. 26,965; Harold L. Novick, Reg. No. 26,011; Suet M. Chong, Reg. No. 38,104; Todd L. Juneau, Reg. No. 40,669; Patricia M. Drost, Reg. No. 29,790; Lee C. Heiman, Reg. No. 41,827; Jerald L. Meyer, Reg. No. 41,194; Joshua B. Goldberg, Reg. No. 44,126; David Milligan, Reg. No. 42,893 and Robert G. Lev, Reg. No. 30,280; David R. Murphy, Reg. No. 22,751; Paul A. Sacher, Reg. No. 43,418; Gregory B. Kang, Reg. No. P-45,273; Scott F. Yarnell, P-45,245; as my attorneys to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected therewith.

Dir. Telephone Calls to:

Gary M. Nath  
(202)775-8383

Send Correspondence to:

Nath & Associates  
Sixth Floor, 1030 15th Street,  
N.W., Washington, D.C. 20005 - USA

We hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by 35 U.S.C. § 112, first paragraph, I/we acknowledge the duty to disclose material information as defined in 37 CFR § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(U.S. Application Serial No.)	(U.S. Filing Date)	(Status--patented, pending, abandoned)
<u>                    </u>	<u>                    </u>	<u>                    </u>
(U.S. Application Serial No.)	(U.S. Filing Date)	(Status--patented, pending, abandoned)
<u>                    </u>	<u>                    </u>	<u>                    </u>

## DECLARATION FOR PATENT APPLICATION

Attorney Docket: 24498

We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor: <sup>1-00</sup> Helge VOELKEL

Inventor's Signature H. Voelkel Date 21/02/2001

Residence: D-98081 Ulm, Germany DEX

Country of Citizenship: Loherstrasse 18/1, D-98081 Ulm, Germany

Post Office Address: \_\_\_\_\_

Full name of second inventor: \_\_\_\_\_

Inventor's Signature \_\_\_\_\_ Date \_\_\_\_\_

Residence: \_\_\_\_\_

Country of Citizenship: \_\_\_\_\_

Post Office Address: \_\_\_\_\_

Full name of third inventor: \_\_\_\_\_

Inventor's Signature \_\_\_\_\_ Date \_\_\_\_\_

Residence: \_\_\_\_\_

Country of Citizenship: \_\_\_\_\_

Post Office Address: \_\_\_\_\_

Full name of fourth inventor: \_\_\_\_\_

Inventor's Signature \_\_\_\_\_ Date \_\_\_\_\_

Residence: \_\_\_\_\_

Country of Citizenship: \_\_\_\_\_

Post Office Address: \_\_\_\_\_

Applicant/Patentee: Helge VOELKEL  
 Serial/Patent No.: 09/744,016 Atty. Dkt No. 24498  
 Filed on/Issued on: \_\_\_\_\_  
 For: Method for screening of modulators of calcineurin activity

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS**  
**(37 CFR § 1.9(d) AND § 1.27(b)) - SMALL BUSINESS CONCERN**

I hereby declare that I am:

- ☐ The owner of the small business concern identified below:  
☐ An official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN Bioinfect GmbH  
 ADDRESS OF CONCERN Loherstrasse 18, DE - 89081 Ulm, Germany

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR § 121.8-18, and reproduced in 37 CFR § 1.9(d), for purposes of paying reduced fees under 35 USC § 41(a) and (b), in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement: (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that the rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled Method for screening of modulators of calcineurin by inventor(s) Helge VOELKEL described in: activity

- ☒ The specification filed herewith  
☐ U.S. Application Serial No. \_\_\_\_\_, filed \_\_\_\_\_  
☐ U.S. Patent No. \_\_\_\_\_, issued \_\_\_\_\_

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR § 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR § 1.9(d) or a non-profit organization under 37 CFR § 1.9(e).

FULL NAME \_\_\_\_\_

ADDRESS \_\_\_\_\_

☐ Individual ☐ Small Business ☐ Non-Profit

\*NOTE: Separate Verified Statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR § 1.27).

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the Issue Fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR § 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 USC § 1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this Verified Statement is directed.

NAME OF PERSON SIGNING Dr. Bernhard Schu  
 TITLE OF PERSON SIGNING Chief Executive Officer  
 ADDRESS OF PERSON SIGNING Kohlgrasse 25, 89073 Ulm / GERMANY

Signature Bioinfect GmbH Date 21/02/2001

Loherstraße 18/1  
 89081 Ulm

## SEQUENCE LISTING

&lt;110&gt; Dr. Völkel, Helge

&lt;120&gt; Method for screening of modulators of calcineurin activity

&lt;130&gt; A34157PCT

&lt;140&gt; PCT/EP99/05220

&lt;141&gt; 1999-07-22

&lt;150&gt; EP98113876

&lt;151&gt; 1998-07-22

&lt;160&gt; 35

&lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 5184

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (662)..(1123)

&lt;223&gt; copper/zinc superoxide dismutase

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1124)..(1849)

&lt;223&gt; enhanced green fluorescent protein

&lt;400&gt; 1

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Trp Gly Ser Ile Lys Gly Leu Thr Glu Gly Leu His Gly Phe His Val	
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His Glu Phe Gly Asp Asn Thr Ala Gly Cys Thr Ser Ala Gly Pro His	
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&lt;210&gt; 5

&lt;211&gt; 239

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 5

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Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile
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Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr
			50				55				60				
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys
			65			70				75					80
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu
			85						90						95
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu
			100					105						110	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly
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Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr
			130				135					140			
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn
			145			150				155					160
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser
			165					170						175	
Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	Gly	Asp	Gly
			180					185					190		
Pro	Val	Leu	Leu	Pro	Asp	Asn	His	Tyr	Leu	Ser	Thr	Gln	Ser	Ala	Leu

- 13 -

195                                      200                                      205  
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 Arg Leu Thr Ala Lys Glu Val Phe Asp Asn Asp Gly Lys Pro Arg Val  
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 Asp Ile Leu Lys Ala His Leu Met Lys Glu Gly Arg Leu Glu Glu Ser  
     50                                      55                                      60  
 Val Ala Leu Arg Ile Ile Thr Glu Gly Ala Ser Ile Leu Arg Gln Glu  
     65                                      70                                      75                                      80  
 Lys Asn Leu Leu Asp Ile Asp Ala Pro Val Thr Val Cys Gly Asp Ile  
                                     85                                      90                                      95  
 His Gly Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly Gly Ser  
                                     100                                      105                                      110  
 Pro Ala Asn Thr Arg Tyr Leu Phe Leu Gly Asp Tyr Val Asp Arg Gly  
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 Tyr Phe Ser Ile Glu Cys Val Leu Tyr Leu Trp Ala Leu Lys Ile Leu  
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 Tyr Pro Lys Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys Arg His  
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 Leu Thr Glu Tyr Phe Thr Phe Lys Gln Glu Cys Lys Ile Lys Tyr Ser  
                                     165                                      170                                      175  
 Glu Arg Val Tyr Asp Ala Cys Met Asp Ala Phe Asp Cys Leu Pro Leu  
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 Ala Ala Leu Met Asn Gln Gln Phe Leu Cys Val His Gly Gly Leu Ser  
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 Pro Glu Ile Asn Thr Leu Asp Asp Ile Arg Lys Leu Asp Arg Phe Lys  
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 Glu Pro Pro Ala Tyr Gly Pro Met Cys Asp Ile Leu Trp Ser Asp Pro  
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 Leu Glu Asp Phe Gly Asn Glu Lys Thr Gln Glu His Phe Thr His Asn  
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&lt;210&gt; 7

&lt;211&gt; 6275

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (613)..(1329)

&lt;223&gt; enhanced green fluorescent protein

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1330)..(2916)

&lt;223&gt; calcineurin A beta

&lt;400&gt; 7

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Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
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His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr
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Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr
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Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu
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Asp	Gly	Ser	Val	Gln	Leu	Ala	Asp	His	Tyr	Gln	Gln	Asn	Thr	Pro	Ile	
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Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Gly	Ala	Asp	Arg	Val	
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Glu	Ala	Pro	Ile	Thr	Val	Cys	Gly	Asp	Ile	His	Gly	Gln	Phe	Phe	Asp	
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gtc	tta	tat	tta	tgg	gtt	ctg	aag	att	cta	tac	cca	agc	aca	tta	ttt	1803
Val	Leu	Tyr	Leu	Trp	Val	Leu	Lys	Ile	Leu	Tyr	Pro	Ser	Thr	Leu	Phe	
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Cys Met Glu Ala Phe Asp Ser Leu Pro Leu Ala Ala Leu Leu Asn Gln	
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Gln Phe Leu Cys Val His Gly Gly Leu Ser Pro Glu Ile His Thr Leu	
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Pro Met Cys Asp Leu Leu Trp Ser Asp Pro Ser Glu Asp Phe Gly Asn	
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Glu Lys Ser Gln Glu His Phe Ser His Asn Thr Val Arg Gly Cys Ser	
495 500 505	
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Tyr Phe Tyr Asn Tyr Pro Ala Val Cys Glu Phe Leu Gln Asn Asn Asn	
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&lt;210&gt; 8

&lt;211&gt; 239

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 8

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Val	Glu	Leu	Asp	Gly	Asp	Val	Asn	Gly	His	Lys	Phe	Ser	Val	Ser	Gly	20	25	30	
Glu	Gly	Glu	Gly	Asp	Ala	Thr	Tyr	Gly	Lys	Leu	Thr	Leu	Lys	Phe	Ile	35	40	45	
Cys	Thr	Thr	Gly	Lys	Leu	Pro	Val	Pro	Trp	Pro	Thr	Leu	Val	Thr	Thr	50	55	60	
Leu	Thr	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Arg	Tyr	Pro	Asp	His	Met	Lys	65	70	75	80
Gln	His	Asp	Phe	Phe	Lys	Ser	Ala	Met	Pro	Glu	Gly	Tyr	Val	Gln	Glu	85	90	95	
Arg	Thr	Ile	Phe	Phe	Lys	Asp	Asp	Gly	Asn	Tyr	Lys	Thr	Arg	Ala	Glu	100	105	110	
Val	Lys	Phe	Glu	Gly	Asp	Thr	Leu	Val	Asn	Arg	Ile	Glu	Leu	Lys	Gly	115	120	125	
Ile	Asp	Phe	Lys	Glu	Asp	Gly	Asn	Ile	Leu	Gly	His	Lys	Leu	Glu	Tyr	130	135	140	
Asn	Tyr	Asn	Ser	His	Asn	Val	Tyr	Ile	Met	Ala	Asp	Lys	Gln	Lys	Asn	145	150	155	160
Gly	Ile	Lys	Val	Asn	Phe	Lys	Ile	Arg	His	Asn	Ile	Glu	Asp	Gly	Ser	165	170	175	

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Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu  
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Gly Ala Ala Ile Leu Arg Arg Glu Lys Thr Met Ile Glu Val Glu Ala  
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Arg Gly Asn His Glu Cys Arg His Leu Thr Glu Tyr Phe Thr Phe Lys  
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Gln Glu Cys Lys Ile Lys Tyr Ser Glu Arg Val Tyr Glu Ala Cys Met  
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Glu Ala Phe Asp Ser Leu Pro Leu Ala Ala Leu Leu Asn Gln Gln Phe  
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Leu Cys Val His Gly Gly Leu Ser Pro Glu Ile His Thr Leu Asp Asp  
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Ile Arg Arg Leu Asp Arg Phe Lys Glu Pro Pro Ala Phe Gly Pro Met

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Cys Asp Leu Leu Trp Ser Asp Pro Ser Glu Asp Phe Gly Asn Glu Lys						
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Ser Gln Glu His Phe Ser His Asn Thr Val Arg Gly Cys Ser Tyr Phe						
		260		265		270
Tyr Asn Tyr Pro Ala Val Cys Glu Phe Leu Gln Asn Asn Asn Leu Leu						
		275		280		285
Ser Ile Ile Arg Ala His Glu Ala Gln Asp Ala Gly Tyr Arg Met Tyr						
		290		295		300
Arg Lys Ser Gln Thr Thr Gly Phe Pro Ser Leu Ile Thr Ile Phe Ser						
		305		310		315
Ala Pro Asn Tyr Leu Asp Val Tyr Asn Asn Lys Ala Ala Val Leu Lys						
		325		330		335
Tyr Glu Asn Asn Val Met Asn Ile Arg Gln Phe Asn Cys Ser Pro His						
		340		345		350
Pro Tyr Trp Leu Pro Asn Phe Met Asp Val Phe Thr Trp Ser Leu Pro						
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Phe Val Gly Glu Lys Val Thr Glu Met Leu Val Asn Val Leu Ser Ile						
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Cys Ser Asp Asp Glu Leu Met Thr Glu Gly Glu Asp Gln Phe Asp Gly						
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Ser Ala Ala Ala Arg Lys Glu Ile Ile Arg Asn Lys Ile Arg Ala Ile						
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Gly Lys Met Ala Arg Val Phe Ser Val Leu Arg Glu Glu Ser Glu Ser						
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Val Leu Ala Gly Gly Arg Gln Thr Leu Gln Ser Ala Thr Val Glu Ala						
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Ile Glu Ala Glu Lys Ala Ile Arg Gly Phe Ser Pro Pro His Arg Ile						
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Cys Ser Phe Glu Glu Ala Lys Gly Leu Asp Arg Ile Asn Glu Arg Met						
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Pro Pro Arg Lys Asp Ala Val Gln Gln Asp Gly Phe Asn Ser Leu Asn						
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&lt;211&gt; 6218

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

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Val Ser Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu  
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Lys Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu  
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Val Thr Thr Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp  
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cgc gcc gag gtg aag ttc gag ggc gac acc ctg gtg aac cgc atc gag 987  
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Leu Glu Tyr Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys				
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Gln Lys Asn Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu				
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Asp Gly Ser Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile				
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tcc gcc ctg agc aaa gac ccc aac gag aag cgc gat cac atg gtc ctg				1275
Ser Ala Leu Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu				
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Tyr Lys Ser Gly Leu Arg Ser Arg Ser Met Ser Gly Arg Arg Phe His				
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Gln Arg Leu Thr Phe Lys Glu Val Phe Glu Asn Gly Lys Pro Lys Val				
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Asp Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Leu Glu Glu Glu				
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Val Ala Leu Lys Ile Ile Asn Asp Gly Ala Ala Ile Leu Arg Gln Glu				
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Lys Thr Met Ile Glu Val Asp Ala Pro Ile Thr Val Cys Gly Asp Ile				
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cat gga caa ttc ttt gac cta atg aag tta ttt gaa gtt gga gga tca				1659
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 Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser

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 Val Tyr Asp Ala Cys Met Glu Thr Phe Asp Cys Leu Pro Leu Ala Ala  
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**SUBSTITUTE SHEET (RULE 26)**

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&lt;211&gt; 153

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&lt;213&gt; Homo sapiens

&lt;400&gt; 14

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Glu Phe Gly Asp Asn Thr Ala Gly Cys Thr Ser Ala Gly Pro His Phe					
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Asn Pro Leu Ser Arg Lys His Gly Gly Pro Lys Asp Glu Glu Arg His					
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Val Gly Asp Leu Gly Asn Val Thr Ala Asp Lys Asp Gly Val Ala Asp					
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Val Ser Ile Glu Asp Ser Val Ile Ser Leu Ser Gly Asp His Cys Ile					
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Ile Gly Arg Thr Leu Val Val His Glu Lys Ala Asp Asp Leu Gly Lys					
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 His Glu Phe Gly Asp Asn Thr Ala Gly Cys Thr Ser Ala Gly Pro His  
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 Phe Asn Pro Leu Ser Arg Lys His Gly Gly Pro Lys Asp Glu Glu Arg  
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 Ile Ile Gly Arg Thr Leu Val Val His Glu Lys Ala Asp Asp Leu Gly  
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 domain), newly generated N-terminus exhibits  
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 Arg Gly Ser His His His His His Gly Ser Met Ser Glu Pro Lys  
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 Ala Ile Asp Pro Lys Leu Ser Thr Thr Asp Arg Val Val Lys Ala Val  
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 Pro Phe Pro Pro Ser His Arg Leu Thr Ala Lys Glu Val Phe Asp Asn  
 35 40 45  
  
 gat gga aaa cct cgt gtg gat atc tta aag gcg cat ctt atg aag gag 309  
 Asp Gly Lys Pro Arg Val Asp Ile Leu Lys Ala His Leu Met Lys Glu  
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 85 90 95  
  
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 Thr Val Cys Gly Asp Ile His Gly Gln Phe Phe Asp Leu Met Lys Leu  
 100 105 110  
  
 ttt gaa gtc ggg gga tct cct gcc aac act cgc tac ctc ttc tta ggg 501  
 Phe Glu Val Gly Gly Ser Pro Ala Asn Thr Arg Tyr Leu Phe Leu Gly  
 115 120 125

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Trp Ala Leu Lys Ile Leu Tyr Pro Lys Thr Leu Phe Leu Leu Arg Gly	
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Asn His Glu Cys Arg His Leu Thr Glu Tyr Phe Thr Phe Lys Gln Glu	
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Cys Lys Ile Lys Tyr Ser Glu Arg Val Tyr Asp Ala Cys Met Asp Ala	
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Phe Asp Cys Leu Pro Leu Ala Ala Leu Met Asn Gln Gln Phe Leu Cys	
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Val His Gly Gly Leu Ser Pro Glu Ile Asn Thr Leu Asp Asp Ile Arg	
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Ala Ala Arg Lys Glu Val Ile Arg Asn Lys Ile Arg Ala Ile Gly Lys	
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 Ala Ser Ile Leu Arg Gln Glu Lys Asn Leu Leu Asp Ile Asp Ala Pro  
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 Cys Val His Gly Gly Leu Ser Pro Glu Ile Asn Thr Leu Asp Asp Ile  
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 Arg Lys Leu Asp Arg Phe Lys Glu Pro Pro Ala Tyr Gly Pro Met Cys  
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Arg Ile Tyr Asp Met Asp Lys Asp Gly Tyr Ile Ser Asn Gly Glu Leu  
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Phe Gln Val Leu Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln  
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Leu Gln Gln Ile Val Asp Lys Thr Ile Ile Asn Ala Asp Lys Asp Gly  
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&lt;223&gt; histidine tagged calcineurin A alpha2

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (1687)..(1728)

&lt;223&gt; ribosomal binding site, multiple cloning site 2

&lt;220&gt;

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&lt;222&gt; (1729)..(2241)

&lt;223&gt; calcineurin B

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&lt;211&gt; 523

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

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Ile Asn Glu Arg Met Pro Pro Arg Arg Asp Ala Met Pro Ser Asp Ala  
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Arg Gly Ser His His His His His Gly Ser Ala Ala Pro Glu Pro  
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Ala Arg Ala Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Gly  
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Thr Ser Glu Glu Val Phe Asp Leu Asp Gly Ile Pro Arg Val Asp Val  
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Ser Ile Glu Cys Val Leu Tyr Leu Trp Val Leu Lys Ile Leu Tyr Pro			
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Ser Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys Arg His Leu Thr			
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Glu Tyr Phe Thr Phe Lys Gln Glu Cys Lys Ile Lys Tyr Ser Glu Arg			
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Pro Ala Phe Gly Pro Met Cys Asp Leu Leu Trp Ser Asp Pro Ser Glu			
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Asp Phe Gly Asn Glu Lys Ser Gln Glu His Phe Ser His Asn Thr Val			
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Ala Gly Tyr Arg Met Tyr Arg Lys Ser Gln Thr Thr Gly Phe Pro Ser			
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Gly Leu Asp Ile His Lys Lys Met Val Val Asp Val			
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- 58 -

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 <212> PRT  
 <213> Homo sapiens

<400> 24

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Pro	Ala	Arg	Ala	Ala	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	20	25	30	
Gly	Ala	Asp	Arg	Val	Val	Lys	Ala	Val	Pro	Phe	Pro	Pro	Thr	His	Arg	35	40	45	
Leu	Thr	Ser	Glu	Glu	Val	Phe	Asp	Leu	Asp	Gly	Ile	Pro	Arg	Val	Asp	50	55	60	
Val	Leu	Lys	Asn	His	Leu	Val	Lys	Glu	Gly	Arg	Val	Asp	Glu	Glu	Ile	65	70	75	80
Ala	Leu	Arg	Ile	Ile	Asn	Glu	Gly	Ala	Ala	Ile	Leu	Arg	Arg	Glu	Lys	85	90	95	
Thr	Met	Ile	Glu	Val	Glu	Ala	Pro	Ile	Thr	Val	Cys	Gly	Asp	Ile	His	100	105	110	
Gly	Gln	Phe	Phe	Asp	Leu	Met	Lys	Leu	Phe	Glu	Val	Gly	Gly	Ser	Pro	115	120	125	
Ala	Asn	Thr	Arg	Tyr	Leu	Phe	Leu	Gly	Asp	Tyr	Val	Asp	Arg	Gly	Tyr	130	135	140	
Phe	Ser	Ile	Glu	Cys	Val	Leu	Tyr	Leu	Trp	Val	Leu	Lys	Ile	Leu	Tyr	145	150	155	160
Pro	Ser	Thr	Leu	Phe	Leu	Leu	Arg	Gly	Asn	His	Glu	Cys	Arg	His	Leu	165	170	175	
Thr	Glu	Tyr	Phe	Thr	Phe	Lys	Gln	Glu	Cys	Lys	Ile	Lys	Tyr	Ser	Glu	180	185	190	
Arg	Val	Tyr	Glu	Ala	Cys	Met	Glu	Ala	Phe	Asp	Ser	Leu	Pro	Leu	Ala	195	200	205	
Ala	Leu	Leu	Asn	Gln	Gln	Phe	Leu	Cys	Val	His	Gly	Gly	Leu	Ser	Pro	210	215	220	
Glu	Ile	His	Thr	Leu	Asp	Asp	Ile	Arg	Arg	Leu	Asp	Arg	Phe	Lys	Glu				



225                      230                      235                      240  
 Pro Pro Ala Phe Gly Pro Met Cys Asp Leu Leu Trp Ser Asp Pro Ser  
                                  245                      250                      255  
 Glu Asp Phe Gly Asn Glu Lys Ser Gln Glu His Phe Ser His Asn Thr  
                                  260                      265                      270  
 Val Arg Gly Cys Ser Tyr Phe Tyr Asn Tyr Pro Ala Val Cys Glu Phe  
                                  275                      280                      285  
 Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala His Glu Ala Gln  
                                  290                      295                      300  
 Asp Ala Gly Tyr Arg Met Tyr Arg Lys Ser Gln Thr Thr Gly Phe Pro  
 305                                   310                      315                      320  
 Ser Leu Ile Thr Ile Phe Ser Ala Pro Asn Tyr Leu Asp Val Tyr Asn  
                                  325                      330                      335  
 Asn Lys Ala Ala Val Leu Lys Tyr Glu Asn Asn Val Met Asn Ile Arg  
                                  340                      345                      350  
 Gln Phe Asn Cys Ser Pro His Pro Tyr Trp Leu Pro Asn Phe Met Asp  
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 Val Phe Thr Trp Ser Leu Pro Phe Val Gly Glu Lys Val Thr Glu Met  
                                  370                      375                      380  
 Leu Val Asn Val Leu Ser Ile Cys Ser Asp Asp Glu Leu Met Thr Glu  
 385                                   390                      395                      400  
 Gly Glu Asp Gln Phe Asp Gly Ser Ala Ala Ala Arg Lys Glu Ile Ile  
                                  405                      410                      415  
 Arg Asn Lys Ile Arg Ala Ile Gly Lys Met Ala Arg Val Phe Ser Val  
                                  420                      425                      430  
 Leu Arg Glu Glu Ser Glu Ser Val Leu Thr Leu Lys Gly Leu Thr Pro  
                                  435                      440                      445  
 Thr Gly Met Leu Pro Ser Gly Val Leu Ala Gly Gly Arg Gln Thr Leu  
                                  450                      455                      460  
 Gln Ser Ala Thr Val Glu Ala Ile Glu Ala Glu Lys Ala Ile Arg Gly  
 465                                   470                      475                      480  
 Phe Ser Pro Pro His Arg Ile Cys Ser Phe Glu Glu Ala Lys Gly Leu  
                                  485                      490                      495  
 Asp Arg Ile Asn Glu Arg Met Pro Pro Arg Lys Asp Ala Val Gln Gln  
                                  500                      505                      510  
 Asp Gly Phe Asn Ser Leu Asn Thr Ala His Ala Thr Glu Asn His Gly  
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 Thr Gly Asn His Thr Ala Gln  
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&lt;210&gt; 25

&lt;211&gt; 170

- 60 -

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 25

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 20 25 30

Asp Asn Ser Gly Ser Leu Ser Val Glu Glu Phe Met Ser Xaa Pro Glu  
 35 40 45

Leu Gln Gln Asn Xaa Leu Val Gln Arg Val Ile Asp Ile Phe Asp Thr  
 50 55 60

Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Xaa Val Ser  
 65 70 75 80

Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe  
 85 90 95

Arg Ile Tyr Asp Met Asp Lys Asp Gly Tyr Ile Ser Asn Gly Glu Leu  
 100 105 110

Phe Gln Xaa Xaa Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln  
 115 120 125

Leu Gln Gln Ile Val Asp Lys Thr Ile Ile Asn Ala Asp Lys Asp Xaa  
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Asp Gly Arg Ile Ser Phe Glu Glu Phe Cys Ala Val Val Xaa Gly Leu  
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Asp Ile His Lys Lys Met Val Val Asp Val  
 165 170

&lt;210&gt; 26

&lt;211&gt; 5611

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (115)..(1776)

&lt;223&gt; histidine tagged calcineurin A beta2

&lt;220&gt;

&lt;221&gt; misc feature

&lt;222&gt; (1777)..(1814)

&lt;223&gt; ribosomal binding site, multiple cloning site 2

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1815)..(2327)

&lt;223&gt; calcineurin B

&lt;400&gt; 26

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attgtgagcg gataacaatt tcacacagaa ttcattaaag aggagaaatt aact atg 117  
Met  
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aga gga tcg cat cac cat cac cat cac gga tcc gcc gcc ccg gag ccg 165  
Arg Gly Ser His His His His His His Gly Ser Ala Ala Pro Glu Pro  
5 10 15

gcc cgg gct gca ccg ccc cca ccc ccg ccc ccg ccg ccc cct ccc ggg 213  
Ala Arg Ala Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Gly  
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Ala Asp Arg Val Val Lys Ala Val Pro Phe Pro Pro Thr His Arg Leu  
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Thr Ser Glu Glu Val Phe Asp Leu Asp Gly Ile Pro Arg Val Asp Val  
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Leu Lys Asn His Leu Val Lys Glu Gly Arg Val Asp Glu Glu Ile Ala  
70 75 80

ctt aga att atc aat gag ggt gct gcc atc ctt cgg aga gag aaa acc 405  
Leu Arg Ile Ile Asn Glu Gly Ala Ala Ile Leu Arg Arg Glu Lys Thr  
85 90 95

atg ata gaa gta gaa gct cca atc aca gtg tgt ggt gac atc cat ggc 453  
Met Ile Glu Val Glu Ala Pro Ile Thr Val Cys Gly Asp Ile His Gly  
100 105 110

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Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly Gly Ser Pro Ala  
115 120 125

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165 170 175

tac cca agc aca tta ttt ctt ctg aga ggc aac cat gaa tgc aga cac 693  
Tyr Pro Ser Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys Arg His  
180 185 190

ctt act gaa tat ttt acc ttt aag cag gaa tgt aaa att aag tat tcg 741  
Leu Thr Glu Tyr Phe Thr Phe Lys Gln Glu Cys Lys Ile Lys Tyr Ser  
195 200 205

gaa aga gtc tat gaa gct tgt atg gaa gct ttt gat agt ttg cct ctt 789  
Glu Arg Val Tyr Glu Ala Cys Met Glu Ala Phe Asp Ser Leu Pro Leu  
210 215 220 225

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cca gaa ata cac	aca ctg gat gat	att agg aga tta	gat aga ttc aaa	885
Pro Glu Ile His	Thr Leu Asp Asp	Ile Arg Arg Leu	Asp Arg Phe Lys	
245	250	255		
gag cca cct gca	ttt gga cca atg	tgt gac ttg tta	tggtcc gat cct	933
Glu Pro Pro Ala	Phe Gly Pro Met	Cys Asp Leu Leu	Trp Ser Asp Pro	
260	265	270		
tct gaa gat ttt	gga aat gaa aaa	tca cag gaa cat	ttt agt cac aat	981
Ser Glu Asp Phe	Gly Asn Glu Lys	Ser Gln Glu His	Phe Ser His Asn	
275	280	285		
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Thr Val Arg Gly	Cys Ser Tyr Phe	Tyr Asn Tyr Pro	Ala Val Cys Glu	
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Phe Leu Gln Asn	Asn Asn Leu Leu	Ser Ile Ile Arg	Ala His Glu Ala	
310	315	320		
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Gln Asp Ala Gly	Tyr Arg Met Tyr	Arg Lys Ser Gln	Thr Thr Gly Phe	
325	330	335		
cct tca tta ata	aca att ttt tcg	gca cct aat tac	tta gat gtc tac	1173
Pro Ser Leu Ile	Thr Ile Phe Ser	Ala Pro Asn Tyr	Leu Asp Val Tyr	
340	345	350		
aat aat aaa gct	gct gta tta aag	tat gaa aat aat	gtg atg aat att	1221
Asn Asn Lys Ala	Ala Val Leu Lys	Tyr Glu Asn Asn	Val Met Asn Ile	
355	360	365		
cga cag ttt aac	tgt tct cca cat	cct tac tgg ttg	cct aat ttt atg	1269
Arg Gln Phe Asn	Cys Ser Pro His	Pro Tyr Trp Leu	Pro Asn Phe Met	
370	375	380	385	
gat gtc ttc acg	tggtct tta ccg	ttt gtt gga gaa	aaa gtg aca gaa	1317
Asp Val Phe Thr	Trp Ser Leu Pro	Phe Val Gly Glu	Lys Val Thr Glu	
390	395	400		
atg ttg gta aat	gtt ctg agt att	tgc tct gat gat	gaa cta atg act	1365
Met Leu Val Asn	Val Leu Ser Ile	Cys Ser Asp Asp	Glu Leu Met Thr	
405	410	415		
gaa ggt gaa gac	cag ttt gat ggt	tca gct gca gcc	cgg aaa gaa atc	1413
Glu Gly Glu Asp	Gln Phe Asp Gly	Ser Ala Ala Ala	Arg Lys Glu Ile	
420	425	430		
ata aga aac aaa	att cga gca att	ggc aag atg gca	aga gtc ttc tct	1461
Ile Arg Asn Lys	Ile Arg Ala Ile	Gly Lys Met Ala	Arg Val Phe Ser	
435	440	445		
gtt ctc agg gag	gag agt gaa agt	gtg ctg aca ctc	aag ggc ctg act	1509
Val Leu Arg Glu	Glu Ser Glu Ser	Val Leu Thr Leu	Lys Gly Leu Thr	
450	455	460	465	
ccc aca ggg atg	ttg cct agt gga	gtg tta gct gga	gga cgg cag acc	1557
Pro Thr Gly Met	Leu Pro Ser Gly	Val Leu Ala Gly	Gly Arg Gln Thr	
470	475	480		

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Leu Gln Ser Ala Thr Val Glu Ala Ile Glu Ala Glu Lys Ala Ile Arg	
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Leu Asp Arg Ile Asn Glu Arg Met Pro Pro Arg Lys Asp Ala Val Gln	
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Met Gly Asn Glu Ala Ser Tyr Pro Leu Glu Met	
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Cys Ser His Phe Asp Xaa Asp Glu Ile Lys Arg Leu Gly Lys Arg Phe	
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Lys Lys Xaa Asp Leu Asp Asn Ser Gly Ser Leu Ser Val Glu Glu Phe	
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Met Ser Xaa Pro Glu Leu Gln Gln Asn Xaa Leu Val Gln Arg Val Ile	
600 605 610	
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Asp Ile Phe Asp Thr Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe	
615 620 625	
att gag ggm gtc tct cag ttc agt gtc aaa gga gat aag gar cag aar	2087
Ile Glu Xaa Val Ser Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys	
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Leu Lys Asp Thr Gln Leu Gln Gln Ile Val Asp Lys Thr Ile Ile Asn	
680 685 690	
gca gat aag gat ggr gat gga aga ata tcc ttt gaa gaa ttc tgt gct	2279
Ala Asp Lys Asp Xaa Asp Gly Arg Ile Ser Phe Glu Glu Phe Cys Ala	
695 700 705	
gtt gta ggy ggc cta gat atc cac aaa aag atg gtg gta gat gtg tga	2327
Val Val Xaa Gly Leu Asp Ile His Lys Lys Met Val Val Asp Val	

710 715 720 725

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 agccagccgg aagggccgag cgcagaagtg gtcctgcaac tttatccgcc tccatccagt 4787  
 ctattaattg ttgccgggaa gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg 4847  
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 gctccggttc ccaacgatca aggcgagtta catgatcccc catgttggtg aaaaaagcgg 4967  
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 gttcgatgta acccactcgt gcacccaact gatcttcagc atcttttact ttcaccagcg 5327  
 tttctgggtg agcaaaaaca ggaaggcaaa atgccgcaaa aaaggaata agggcgacac 5387  
 ggaaatgttg aatactcata ctcttccttt ttcaatatta ttgaagcatt tatcagggtt 5447  
 attgtctcat gagcggatac atatttgaat gtatttagaa aaataaacia ataggggttc 5507  
 cgcgcacatt tccccgaaaa gtgccacctg acgtctaaga aaccattatt atcatgacat 5567  
 taacctataa aaataggcgt atcacgaggc cctttcgtct tcac 5611

&lt;210&gt; 27

&lt;211&gt; 553

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 27

Met Arg Gly Ser His His His His His His Gly Ser Ala Ala Pro Glu  
 1 5 10 15

Pro Ala Arg Ala Ala Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro  
 20 25 30

Gly Ala Asp Arg Val Val Lys Ala Val Pro Phe Pro Pro Thr His Arg  
 35 40 45  
 Leu Thr Ser Glu Glu Val Phe Asp Leu Asp Gly Ile Pro Arg Val Asp  
 50 55 60  
 Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Val Asp Glu Glu Ile  
 65 70 75 80  
 Ala Leu Arg Ile Ile Asn Glu Gly Ala Ala Ile Leu Arg Arg Glu Lys  
 85 90 95  
 Thr Met Ile Glu Val Glu Ala Pro Ile Thr Val Cys Gly Asp Ile His  
 100 105 110  
 Gly Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly Gly Ser Pro  
 115 120 125  
 Ala Asn Thr Arg Tyr Leu Phe Leu Gly Asp Tyr Val Asp Arg Gly Tyr  
 130 135 140  
 Phe Ser Ile Glu His Val Leu Gly Thr Glu Asp Ile Ser Ile Asn Pro  
 145 150 155 160  
 His Asn Asn Ile Asn Glu Cys Val Leu Tyr Leu Trp Val Leu Lys Ile  
 165 170 175  
 Leu Tyr Pro Ser Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys Arg  
 180 185 190  
 His Leu Thr Glu Tyr Phe Thr Phe Lys Gln Glu Cys Lys Ile Lys Tyr  
 195 200 205  
 Ser Glu Arg Val Tyr Glu Ala Cys Met Glu Ala Phe Asp Ser Leu Pro  
 210 215 220  
 Leu Ala Ala Leu Leu Asn Gln Gln Phe Leu Cys Val His Gly Gly Leu  
 225 230 235 240  
 Ser Pro Glu Ile His Thr Leu Asp Asp Ile Arg Arg Leu Asp Arg Phe  
 245 250 255  
 Lys Glu Pro Pro Ala Phe Gly Pro Met Cys Asp Leu Leu Trp Ser Asp  
 260 265 270  
 Pro Ser Glu Asp Phe Gly Asn Glu Lys Ser Gln Glu His Phe Ser His  
 275 280 285  
 Asn Thr Val Arg Gly Cys Ser Tyr Phe Tyr Asn Tyr Pro Ala Val Cys  
 290 295 300  
 Glu Phe Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala His Glu  
 305 310 315 320  
 Ala Gln Asp Ala Gly Tyr Arg Met Tyr Arg Lys Ser Gln Thr Thr Gly  
 325 330 335  
 Phe Pro Ser Leu Ile Thr Ile Phe Ser Ala Pro Asn Tyr Leu Asp Val  
 340 345 350  
 Tyr Asn Asn Lys Ala Ala Val Leu Lys Tyr Glu Asn Asn Val Met Asn



- 67 -

355	360	365
Ile Arg Gln Phe Asn Cys Ser Pro His Pro Tyr Trp Leu Pro Asn Phe 370 375 380		
Met Asp Val Phe Thr Trp Ser Leu Pro Phe Val Gly Glu Lys Val Thr 385 390 395 400		
Glu Met Leu Val Asn Val Leu Ser Ile Cys Ser Asp Asp Glu Leu Met 405 410 415		
Thr Glu Gly Glu Asp Gln Phe Asp Gly Ser Ala Ala Ala Arg Lys Glu 420 425 430		
Ile Ile Arg Asn Lys Ile Arg Ala Ile Gly Lys Met Ala Arg Val Phe 435 440 445		
Ser Val Leu Arg Glu Glu Ser Glu Ser Val Leu Thr Leu Lys Gly Leu 450 455 460		
Thr Pro Thr Gly Met Leu Pro Ser Gly Val Leu Ala Gly Gly Arg Gln 465 470 475 480		
Thr Leu Gln Ser Ala Thr Val Glu Ala Ile Glu Ala Glu Lys Ala Ile 485 490 495		
Arg Gly Phe Ser Pro Pro His Arg Ile Cys Ser Phe Glu Glu Ala Lys 500 505 510		
Gly Leu Asp Arg Ile Asn Glu Arg Met Pro Pro Arg Lys Asp Ala Val 515 520 525		
Gln Gln Asp Gly Phe Asn Ser Leu Asn Thr Ala His Ala Thr Glu Asn 530 535 540		
His Gly Thr Gly Asn His Thr Ala Gln 545 550		

&lt;210&gt; 28

&lt;211&gt; 170

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 28

Met Gly Asn Glu Ala Ser Tyr Pro Leu Glu Met Cys Ser His Phe Asp 1 5 10 15
Xaa Asp Glu Ile Lys Arg Leu Gly Lys Arg Phe Lys Lys Xaa Asp Leu 20 25 30
Asp Asn Ser Gly Ser Leu Ser Val Glu Glu Phe Met Ser Xaa Pro Glu 35 40 45
Leu Gln Gln Asn Xaa Leu Val Gln Arg Val Ile Asp Ile Phe Asp Thr 50 55 60
Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Xaa Val Ser 65 70 75 80
Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe 85 90 95

Arg Ile Tyr Asp Met Asp Lys Asp Gly Tyr Ile Ser Asn Gly Glu Leu  
 100 105 110

Phe Gln Xaa Xaa Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln  
 115 120 125

Leu Gln Gln Ile Val Asp Lys Thr Ile Ile Asn Ala Asp Lys Asp Xaa  
 130 135 140

Asp Gly Arg Ile Ser Phe Glu Glu Phe Cys Ala Val Val Xaa Gly Leu  
 145 150 155 160

Asp Ile His Lys Lys Met Val Val Asp Val  
 165 170

<210> 29  
 <211> 5498  
 <212> DNA  
 <213> Homo sapiens

<220>  
 <221> CDS  
 <222> (115)..(1659)  
 <223> histidine tagged calcineurin A gamma1

<220>  
 <221> misc\_feature  
 <222> (1660)..(1701)  
 <223> ribosomal binding site, multiple cloning site 2

<220>  
 <221> CDS  
 <222> (1702)..(2214)  
 <223> calcineurin B

<400> 29  
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attgtgagcg gataacaatt tcacacagaa ttcattaaag aggagaaatt aact atg 117  
 Met  
 1

aga gga tcg cat cac cat cac cat cac gga tcc atg tcc ggg agg cgc 165  
 Arg Gly Ser His His His His His Gly Ser Met Ser Gly Arg Arg  
 5 10 15

ttc cac ctc tcc acc acc gac cgc gtc atc aaa gct gtc ccc ttt cct 213  
 Phe His Leu Ser Thr Thr Asp Arg Val Ile Lys Ala Val Pro Phe Pro  
 20 25 30

cca acc caa cgg ctt act ttc aag gaa gta ttt gag aat ggg aaa cct 261  
 Pro Thr Gln Arg Leu Thr Phe Lys Glu Val Phe Glu Asn Gly Lys Pro  
 35 40 45

aaa gtt gat gtt tta aaa aac cat ttg gta aag gaa gga cga ctg gaa 309  
 Lys Val Asp Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Leu Glu  
 50 55 60 65

gag gaa gta gcc tta aag ata atc aat gat ggg gct gcc atc ctg agg 357

Glu Glu Val Ala Leu Lys Ile Ile Asn Asp Gly Ala Ala Ile Leu Arg  
 70 75 80  
 caa gag aag act atg ata gaa gta gat gct cca atc aca gta tgt ggt 405  
 Gln Glu Lys Thr Met Ile Glu Val Asp Ala Pro Ile Thr Val Cys Gly  
 85 90 95  
 gat att cat gga caa ttc ttt gac cta atg aag tta ttt gaa gtt gga 453  
 Asp Ile His Gly Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly  
 100 105 110  
 gga tca cct agt aac aca cgc tac ctc ttt ctg ggt gac tat gtg gac 501  
 Gly Ser Pro Ser Asn Thr Arg Tyr Leu Phe Leu Gly Asp Tyr Val Asp  
 115 120 125  
 aga ggc tat ttc agt ata gag tgt gtg ctg tat tta tgg agt tta aag 549  
 Arg Gly Tyr Phe Ser Ile Glu Cys Val Leu Tyr Leu Trp Ser Leu Lys  
 130 135 140 145  
 att aat cat ccc aaa aca ttg ttt ctg ctt cgg gga aat cat gaa tgc 597  
 Ile Asn His Pro Lys Thr Leu Phe Leu Leu Arg Gly Asn His Glu Cys  
 150 155 160  
 agg cat ctt aca gac tat ttc acc ttc aaa cag gaa tgt cga atc aaa 645  
 Arg His Leu Thr Asp Tyr Phe Thr Phe Lys Gln Glu Cys Arg Ile Lys  
 165 170 175  
 tat tcg gaa cag gtg tat gat gcc tgt atg gag aca ttt gac tgt ctt 693  
 Tyr Ser Glu Gln Val Tyr Asp Ala Cys Met Glu Thr Phe Asp Cys Leu  
 180 185 190  
 cct ctt gct gcc ctc tta aac cag cag ttt ctc tgt gta cat gga gga 741  
 Pro Leu Ala Ala Leu Leu Asn Gln Gln Phe Leu Cys Val His Gly Gly  
 195 200 205  
 atg tca cct gaa att act tct tta gat gac att agg aaa tta gac agg 789  
 Met Ser Pro Glu Ile Thr Ser Leu Asp Asp Ile Arg Lys Leu Asp Arg  
 210 215 220 225  
 ttt acg gaa cct ccc gcc ttt gga cct gtg tgt gac ctg ctt tgg tct 837  
 Phe Thr Glu Pro Pro Ala Phe Gly Pro Val Cys Asp Leu Leu Trp Ser  
 230 235 240  
 gat ccc tca gag gat tat ggc aat gag aag acc ttg gag cac tat acc 885  
 Asp Pro Ser Glu Asp Tyr Gly Asn Glu Lys Thr Leu Glu His Tyr Thr  
 245 250 255  
 cac aac act gtc cga ggg tgc tct tat ttc tac agt tac cct gca gtt 933  
 His Asn Thr Val Arg Gly Cys Ser Tyr Phe Tyr Ser Tyr Pro Ala Val  
 260 265 270  
 tgt gaa ttt ttg cag aac aat aat tta cta tca att atc aga gcc cat 981  
 Cys Glu Phe Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala His  
 275 280 285  
 gaa gcc caa gat gct ggg tat cga atg tac agg aag agc caa gcc aca 1029  
 Glu Ala Gln Asp Ala Gly Tyr Arg Met Tyr Arg Lys Ser Gln Ala Thr  
 290 295 300 305  
 ggc ttt cca tca ctt att aca att ttc tct gcc ccc aat tac cta gat 1077  
 Gly Phe Pro Ser Leu Ile Thr Ile Phe Ser Ala Pro Asn Tyr Leu Asp  
 310 315 320

gtc tat aac aat aaa gct gct gtg ttg aaa tat gaa aac aat gtc atg 1125  
 Val Tyr Asn Asn Lys Ala Ala Val Leu Lys Tyr Glu Asn Asn Val Met  
 325 330 335

aat atc agg cag ttt aac tgt tct cca cac ccc tac tgg ctt cca aac 1173  
 Asn Ile Arg Gln Phe Asn Cys Ser Pro His Pro Tyr Trp Leu Pro Asn  
 340 345 350

ttt atg gat gtt ttc aca tgg tct ttg cct ttt gtt ggg gaa aaa gtc 1221  
 Phe Met Asp Val Phe Thr Trp Ser Leu Pro Phe Val Gly Glu Lys Val  
 355 360 365

aca gag atg ctg gta aat gtg ctc aac ata tgc tct gat gac gaa ctg 1269  
 Thr Glu Met Leu Val Asn Val Leu Asn Ile Cys Ser Asp Asp Glu Leu  
 370 375 380 385

att tct gat gat gaa gca gaa gga agc act aca gtt cgt aag gag atc 1317  
 Ile Ser Asp Asp Glu Ala Glu Gly Ser Thr Thr Val Arg Lys Glu Ile  
 390 395 400

atc agg aat aag atc aga gcc att ggg aag atg gca cgg gtc ttt tca 1365  
 Ile Arg Asn Lys Ile Arg Ala Ile Gly Lys Met Ala Arg Val Phe Ser  
 405 410 415

att ctt cgg caa gaa agt gag agt gtg ctg act ctc aag ggc ctg act 1413  
 Ile Leu Arg Gln Glu Ser Glu Ser Val Leu Thr Leu Lys Gly Leu Thr  
 420 425 430

ccc aca ggc aca ctc cct ctg ggc gtc ctc tca gga ggc aag cag act 1461  
 Pro Thr Gly Thr Leu Pro Leu Gly Val Leu Ser Gly Gly Lys Gln Thr  
 435 440 445

atc gag aca gcc atc aga ggg ttc tgc ctt cag cac aag atc cgg agt 1509  
 Ile Glu Thr Ala Ile Arg Gly Phe Ser Leu Gln His Lys Ile Arg Ser  
 450 455 460 465

ttt gaa gaa gcg cga ggt ctg gac cga att aat gag cga atg cca ccc 1557  
 Phe Glu Glu Ala Arg Gly Leu Asp Arg Ile Asn Glu Arg Met Pro Pro  
 470 475 480

cga aag gat agc ata tac cct ggt ggg cca atg aaa tct gta acc tca 1605  
 Arg Lys Asp Ser Ile Tyr Pro Gly Gly Pro Met Lys Ser Val Thr Ser  
 485 490 495

gca cac tca cat gct gcg cac agg agc gac caa ggg aag aaa gcc cat 1653  
 Ala His Ser His Ala Ala His Arg Ser Asp Gln Gly Lys Lys Ala His  
 500 505 510

tca tga ttaactaggg taccccgggg taccaaagag gagaaattaa ct atg gga 1707  
 Ser Met Gly  
 515

aat gag gca agt tat cct ttg gaa atg tgc tca cac ttt gat gca gat 1755  
 Asn Glu Ala Ser Tyr Pro Leu Glu Met Cys Ser His Phe Asp Ala Asp  
 520 525 530

gaa att aaa agg cta gga aag aga ttt aag aag ctc gat ttg gac aat 1803  
 Glu Ile Lys Arg Leu Gly Lys Arg Phe Lys Lys Leu Asp Leu Asp Asn  
 535 540 545

tct ggt tct ttg agt gtg gaa gag ttc atg tct cta cct gag tta caa 1851

Ser Gly Ser Leu Ser Val Glu Glu Phe Met Ser Leu Pro Glu Leu Gln  
 550 555 560 565

cag aat ccc tta gta cag cga gta ata gat ata ttc gac aca gat ggg 1899  
 Gln Asn Pro Leu Val Gln Arg Val Ile Asp Ile Phe Asp Thr Asp Gly  
 570 575 580

aat gga gaa gta gac ttt aaa gag ttc att gag gga gtc tct cag ttc 1947  
 Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Gly Val Ser Gln Phe  
 585 590 595

agt gtc aaa gga gat aag gaa cag aag ttg agg ttt gct ttc cgt atc 1995  
 Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe Arg Ile  
 600 605 610

tat gac atg gat aaa gac ggc tat att tcc aat ggg gaa ctc ttc cag 2043  
 Tyr Asp Met Asp Lys Asp Gly Tyr Ile Ser Asn Gly Glu Leu Phe Gln  
 615 620 625

gtg cta aag atg atg gtg ggg aac aat ctg aaa gat aca cag tta cag 2091  
 Val Leu Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln Leu Gln  
 630 635 640 645

caa att gta gac aaa acc ata ata aat gca gat aag gat ggg gat gga 2139  
 Gln Ile Val Asp Lys Thr Ile Ile Asn Ala Asp Lys Asp Gly Asp Gly  
 650 655 660

aga ata tcc ttt gaa gaa ttc tgt gct gtt gta ggc ggc cta gat atc 2187  
 Arg Ile Ser Phe Glu Glu Phe Cys Ala Val Val Gly Gly Leu Asp Ile  
 665 670 675

cac aaa aag atg gtg gta gat gtg tga ttaattagaa gcttaattag 2234  
 His Lys Lys Met Val Val Asp Val  
 680 685

ctgagcttgg actcctgttg atagatccag taatgacctc agaactccat ctggatttgt 2294

tcagaacgct cgggttgcgc cgggcgtttt ttattgtgga gaatccaagc tagcttggcg 2354

agattttcag gagctaagga agctaaaatg gagaaaaaaaa tcaactggata taccaccgtt 2414

gatatatccc aatggcatcg taaagaacat tttgaggcat ttcagtcagt tgctcaatgt 2474

acctataacc agaccgttca gctggatatt acggcctttt taaagaccgt aaagaaaaat 2534

aagcacaagt tttatccggc ctttattcac attcttgccc gctgatgaa tgctcatccg 2594

gaatttcgta tggcaatgaa agacggtgag ctggtgatat gggatagtgt tcacccttgt 2654

tacaccgttt tccatgagca aactgaaacg ttttcatcgc tctggagtga ataccacgac 2714

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gcctatttcc ctaaagggtt tattgagaat atgtttttcg tctcagccaa tccctgggtg 2834

agtttcacca gttttgattt aaacgtggcc aatatggaca acttcttcgc ccccgttttc 2894

accatgggca aatattatac gcaaggcgac aaggtgctga tgccgctggc gattcaggtt 2954

catcatgccc tctgtgatgg cttccatgtc ggcagaatgc ttaatgaatt acaacagtac 3014

tgcatgagt ggcagggcgg ggcgtaattt ttttaaggca gttattgggtg cccttaaacg 3074

cctgggggtaa tgactctcta gcttgaggca tcaaataaaa cgaaaggctc agtcgaaaga 3134  
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tatcgtcttg agtccaacc ggtaagacac gacttatcgc cactggcagc agccactggt 4034  
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gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg ttgttgccat tgctacaggc 4754  
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aattctctta ctgtcatgcc atccgtaaga tgcttttctg tgactgggtga gtactcaacc 4994  
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gcacccaact gatcttcagc atcttttact ttcaccagcg tttctgggtg agcaaaaaaca 5234  
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ctcttccttt ttcaatatta ttgaagcatt tatcagggtt attgtctcat gagcggatac 5354  
atatttgaat gtatttagaa aaataaacia ataggggttc cgcgcacatt tccccgaaaa 5414  
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atcacgagge cctttcgtct tcac 5498

<210> 30  
<211> 514  
<212> PRT  
<213> Homo sapiens

<400> 30  
Met Arg Gly Ser His His His His His His Gly Ser Met Ser Gly Arg  
1 5 10 15  
Arg Phe His Leu Ser Thr Thr Asp Arg Val Ile Lys Ala Val Pro Phe  
20 25 30  
Pro Pro Thr Gln Arg Leu Thr Phe Lys Glu Val Phe Glu Asn Gly Lys  
35 40 45  
Pro Lys Val Asp Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Leu  
50 55 60  
Glu Glu Glu Val Ala Leu Lys Ile Ile Asn Asp Gly Ala Ala Ile Leu  
65 70 75 80  
Arg Gln Glu Lys Thr Met Ile Glu Val Asp Ala Pro Ile Thr Val Cys  
85 90 95  
Gly Asp Ile His Gly Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val  
100 105 110  
Gly Gly Ser Pro Ser Asn Thr Arg Tyr Leu Phe Leu Gly Asp Tyr Val  
115 120 125  
Asp Arg Gly Tyr Phe Ser Ile Glu Cys Val Leu Tyr Leu Trp Ser Leu  
130 135 140  
Lys Ile Asn His Pro Lys Thr Leu Phe Leu Leu Arg Gly Asn His Glu  
145 150 155 160  
Cys Arg His Leu Thr Asp Tyr Phe Thr Phe Lys Gln Glu Cys Arg Ile  
165 170 175  
Lys Tyr Ser Glu Gln Val Tyr Asp Ala Cys Met Glu Thr Phe Asp Cys

180 185 190  
 Leu Pro Leu Ala Ala Leu Leu Asn Gln Gln Phe Leu Cys Val His Gly  
 195 200 205  
 Gly Met Ser Pro Glu Ile Thr Ser Leu Asp Asp Ile Arg Lys Leu Asp  
 210 215 220  
 Arg Phe Thr Glu Pro Pro Ala Phe Gly Pro Val Cys Asp Leu Leu Trp  
 225 230 235 240  
 Ser Asp Pro Ser Glu Asp Tyr Gly Asn Glu Lys Thr Leu Glu His Tyr  
 245 250 255  
 Thr His Asn Thr Val Arg Gly Cys Ser Tyr Phe Tyr Ser Tyr Pro Ala  
 260 265 270  
 Val Cys Glu Phe Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala  
 275 280 285  
 His Glu Ala Gln Asp Ala Gly Tyr Arg Met Tyr Arg Lys Ser Gln Ala  
 290 295 300  
 Thr Gly Phe Pro Ser Leu Ile Thr Ile Phe Ser Ala Pro Asn Tyr Leu  
 305 310 315 320  
 Asp Val Tyr Asn Asn Lys Ala Ala Val Leu Lys Tyr Glu Asn Asn Val  
 325 330 335  
 Met Asn Ile Arg Gln Phe Asn Cys Ser Pro His Pro Tyr Trp Leu Pro  
 340 345 350  
 Asn Phe Met Asp Val Phe Thr Trp Ser Leu Pro Phe Val Gly Glu Lys  
 355 360 365  
 Val Thr Glu Met Leu Val Asn Val Leu Asn Ile Cys Ser Asp Asp Glu  
 370 375 380  
 Leu Ile Ser Asp Asp Glu Ala Glu Gly Ser Thr Thr Val Arg Lys Glu  
 385 390 395 400  
 Ile Ile Arg Asn Lys Ile Arg Ala Ile Gly Lys Met Ala Arg Val Phe  
 405 410 415  
 Ser Ile Leu Arg Gln Glu Ser Glu Ser Val Leu Thr Leu Lys Gly Leu  
 420 425 430  
 Thr Pro Thr Gly Thr Leu Pro Leu Gly Val Leu Ser Gly Gly Lys Gln  
 435 440 445  
 Thr Ile Glu Thr Ala Ile Arg Gly Phe Ser Leu Gln His Lys Ile Arg  
 450 455 460  
 Ser Phe Glu Glu Ala Arg Gly Leu Asp Arg Ile Asn Glu Arg Met Pro  
 465 470 475 480  
 Pro Arg Lys Asp Ser Ile Tyr Pro Gly Gly Pro Met Lys Ser Val Thr  
 485 490 495  
 Ser Ala His Ser His Ala Ala His Arg Ser Asp Gln Gly Lys Lys Ala  
 500 505 510



- 75 -

His Ser

&lt;210&gt; 31

&lt;211&gt; 170

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 31

Met Gly Asn Glu Ala Ser Tyr Pro Leu Glu Met Cys Ser His Phe Asp  
 1 5 10 15

Ala Asp Glu Ile Lys Arg Leu Gly Lys Arg Phe Lys Lys Leu Asp Leu  
 20 25 30

Asp Asn Ser Gly Ser Leu Ser Val Glu Glu Phe Met Ser Leu Pro Glu  
 35 40 45

Leu Gln Gln Asn Pro Leu Val Gln Arg Val Ile Asp Ile Phe Asp Thr  
 50 55 60

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Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe  
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Phe Gln Val Leu Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln  
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Asp Ile His Lys Lys Met Val Val Asp Val  
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&lt;210&gt; 32

&lt;211&gt; 5528

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (115)..(1689)

&lt;223&gt; histidine tagged calcineurin A gamma2

&lt;220&gt;

&lt;221&gt; misc\_feature

&lt;222&gt; (1690)..(1731)

&lt;223&gt; ribosomal binding site, multiple cloning site 2

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1732)..(2244)

&lt;223&gt; calcineurin B

&lt;220&gt;

&lt;221&gt; variation

&lt;222&gt; (1474)..(1503)

<223> splicevariant: substitution with ACAGTAGAAG  
 CGGTAGAGGC CCGGGAAGCC, interaction domain with  
 cytoskeleton, death-domain homolog, stomatin  
 homolog

&lt;400&gt; 32

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                                         Met
                                         1

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Phe His Leu Ser Thr Thr Asp Arg Val Ile Lys Ala Val Pro Phe Pro
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cca acc caa cgg ctt act ttc aag gaa gta ttt gag aat ggg aaa cct      261
Pro Thr Gln Arg Leu Thr Phe Lys Glu Val Phe Glu Asn Gly Lys Pro
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Lys Val Asp Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Leu Glu
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Asp Ile His Gly Gln Phe Phe Asp Leu Met Lys Leu Phe Glu Val Gly
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Cys	Glu	Phe	Leu	Gln	Asn	Asn	Asn	Leu	Leu	Ser	Ile	Ile	Arg	Ala	His	
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Asn	Ile	Arg	Gln	Phe	Asn	Cys	Ser	Pro	His	Pro	Tyr	Trp	Leu	Pro	Asn	
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Pro Gly Gly Pro Met Lys Ser Val Thr Ser Ala His Ser His Ala Ala	
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Asp Gly Tyr Ile Ser Asn Gly Glu Leu Phe Gln Val Leu Lys Met Met	
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&lt;211&gt; 524

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 33

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Pro Lys Val Asp Val Leu Lys Asn His Leu Val Lys Glu Gly Arg Leu
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Glu Glu Glu Val Ala Leu Lys Ile Ile Asn Asp Gly Ala Ala Ile Leu
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Lys Ile Asn His Pro Lys Thr Leu Phe Leu Leu Arg Gly Asn His Glu
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Cys Arg His Leu Thr Asp Tyr Phe Thr Phe Lys Gln Glu Cys Arg Ile
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Lys Tyr Ser Glu Gln Val Tyr Asp Ala Cys Met Glu Thr Phe Asp Cys
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Gly Met Ser Pro Glu Ile Thr Ser Leu Asp Asp Ile Arg Lys Leu Asp
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Val Cys Glu Phe Leu Gln Asn Asn Asn Leu Leu Ser Ile Ile Arg Ala
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Thr Gly Phe Pro Ser Leu Ile Thr Ile Phe Ser Ala Pro Asn Tyr Leu

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 Gly Leu Asp Arg Ile Asn Glu Arg Met Pro Pro Arg Lys Asp Ser Ile  
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 Asp Gly Asn Gly Glu Val Asp Phe Lys Glu Phe Ile Glu Gly Val Ser  
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Gln Phe Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe  
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 Arg Ile Tyr Asp Met Asp Lys Asp Gly Tyr Ile Ser Asn Gly Glu Leu  
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 Phe Gln Val Leu Lys Met Met Val Gly Asn Asn Leu Lys Asp Thr Gln  
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